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THE COLORIMETRIC DETERMINATION OF HYDROGEN ION CONCENTRATION AND ITS APPLICATIONS IN BACTERIOLOGY[1]

PART I

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SECTION I. INTRODUCTION

In the classic work of Pasteur are to be found many important suggestions as to the manner in which microbial life responds to the acidity of culture media. As an instance we may recall the opening chapter of Studies on Fermentation, in which Pasteur (1879) notes that the relatively high acidity of must favors a natural alcoholic fermentation in wine, while the low acidity of wort makes the brewing of beer more difficult. Likewise, according to Pasteur, it is the difference in the acidity of wort and must which determines in large measure their relative susceptibility to "disease." Today in the manufacture of beer, certain of the processes are being controlled by following the hydrogen ion concentration; and in judging the quality of wines the determination of their hydrogen ion concentrations is considered an important test.

In his criticism of Duval's theory that yeasts may metamorphose to "lactic ferments," Pasteur (1879), with characteristic glee, pointed out that in Duval's experiment the medium had a reaction favorable to the "lactic ferments" and unfavorable to the yeasts. Consequently, had the inoculation been mixed, the "lactic ferments" would have outgrown the yeasts. Although the direct bearing of this critique is now only historical, the

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remark itself has lost none of its significance. Dalyell (1914) says,

> A constant difficulty in bacteriological examination of feces is the selective action of artificial media which allows certain organisms to outgrow others. For example, if a portion of fecal material be inoculated (a) into ordinary nutrient bouillon of alkaline reaction, (b) into such bouillon containing a large amount of bile, and (c) into nutrient bouillon acidified with 1 per cent of acetic acid, and if the sediments of the three tubes be examined, each will show a different microscopic picture, and none will conform to that of a smear made directly from feces.

Even the influence of acidity upon the effective temperature of the process we have come to call pasteurization did not escape the notice of Pasteur (1879). In a brief paper presented at the Philadelphia meeting of the Society of American Bacteriologists in 1914, one of us (Clark 1915 a) remarked that in cellular destruction temperature is to be considered as an accelerating *condition*, and showed that, among the active *agents* concerned, the concentration of the hydrogen ions in a medium may be of great significance. In a broad sense the term "thermal death point," as Pasteur showed, has no meaning when considered apart from the agents or the processes involved in cellular destruction at high temperatures, although it may have a definite practical significance in the study of such problems as the pasteurization of a relatively constant medium such as milk.

Since Pasteur's time, the influence of acidity and alkalinity upon the activity of microörganisms has been studied in many details and from many points of view. An adequate review of the literature bearing upon this subject is a task for which we must admit our incapacity, for so many of the significant observations which we have noted were gleaned from papers on other themes that we feel sure we have overlooked much truly important work. To deal only with those investigations which have been undertaken with the more modern physical chemist's point of view would be to a certain extent unjust, for many of these are of less real value than the observations made from other view points. A judicious review would also make exorbitant

demands upon space, since many aspects of the subject are worthy of sympathetic treatment. For instance, in considering the introduction of the titrimetric method of adjusting the "reaction" of culture media, it should not only be pointed out that the procedure was applied to a problem where it found no fundamental theoretical justification; it should also be recalled that this method did make possible a certain degree of uniformity in the adjustment of important and well studied media, and that the mere introduction of a scale, however inadequate its unit, was an advance.

To realize the scope and diversity of but one class of problems in which the acidity of culture media is involved, we need only recall those numerous cases in which the acid produced by one organism, has, or is alleged to have, an inhibitory effect upon other organisms. The use of vinegar as a food preservative is essentially a case in point. Vinegar is protected from the successful invasion of the hosts of the microscopic world by its high acidity, and there is no reason to doubt that less acid liquids, though fortified less strongly, resist invasion in precisely the same way. Where indeed shall we place the line of demarcation? Fruit juices, because of their acidity, are perhaps as effectively immune to successful attack by *certain* organisms as the strongest vinegar; and the acidity of cheese probably protects it against those kinds of bacterial action that would make it unfit for human food. The suppression of putrefactive, pathogenic and coliform organisms (in laboratory cultures) by bacteria of the acidophylic bulgaricus type has been widely discussed—very largely because it forms a basic argument for the Metchnikoff treatment of certain intestinal conditions, but also because it is intimately associated with the manufacture of cheese, rennet, silage and several acid fermented foods in which organisms of the bulgaricus type are dominant.

We need not discuss the conflicting conclusions which have been reached in the study of these and similar problems, but we may note that almost without exception the study of the influence of acidity upon microbial activity has been approached with no other method than the determination of "titratable

acidity." The fundamental objections to this method when applied to physiological problems have been pointed out by Henderson (1909), Sörensen (1909 a and b), Michaelis (1914 b) and others, and with special reference to bacteriological culture media by Clark (1915 e).

It may be reiterated here that the titrimetric method, designed originally for the quantitative estimation of strong acids and bases, can not be applied to complex mixtures of very weak acidic and basic groups such as are found in the constituents of most culture media. In so far as the method is used to determine the "free acid" or to adjust to a certain degree of "free acid" it is an absolute failure when applied to culture media.

There is however, an even more fundamental reason why the titrimetric method is inappropriate. Two media adjusted to the same "degree of titratable acidity" may have widely divergent hydrogen ion concentrations as shown by Clark (1915 e). Now, if it is granted that the bacterial enzymes are comparable with other better known enzymes, and, if the validity of such classical work as that of Sörensen (1909) is admitted, then it must be supposed that the hydrogen ion concentration of a medium rather than the titratable acidity influences the enzymatic activity of bacteria and their relatives. If, furthermore, it is granted that the protoplasmic structure of the bacterial cell, or of its membrane, responds to any extent like protein solutions, then there is reason to suppose that the stability of this structure is to some degree dependent upon the hydrogen ion concentration about it. If the membrane is to any degree a mosaic structure with interfaces as sensitive to "reaction" as are those of certain emulsions, then the hydrogen ion concentration of the media must be taken into consideration when dealing with the penetration of food or poison.

Such considerations furnish a more fundamental point of view from which to regard in a new light many of the problems of bacteriology. Using such a view as a working hypothesis, it might safely be postulated that there should be hydrogen ion concentrations which limit the activity of one or another organism. A few of the earlier papers touched on this side of the

subject. Lazarus (1908), for instance, adjusted her media with the aid of various indicators and determined roughly the hydrogen ion concentrations which limited bacterial growth.

In 1912 Michaelis and Marcora, with more accurate methods and a clearer conception of the problem, demonstrated that *B. coli* is limited in its acid fermentation of sugars upon reaching a rather definite hydrogen ion concentration.

A more extensive research by one of us (Clark 1915 c) confirmed the conclusion of Michaelis and Marcora in its essential point. It was mentioned at the time that a similar phenomenon had been observed with a few cultures of streptococci. This has since been abundantly confirmed by Ayers (1916) in his study of some 200 cultures. These results with bacteria of the colon and streptococcus groups together with some preliminary work with other groups of organisms have shown that by determining the final hydrogen ion concentrations of sugar-fermenting cultures, instead of titrating such cultures, values are obtained which are far more constant, and more reproducible in different media, of great diagnostic worth, and of auspicious significance.

A further instance of the use of hydrogen ion determinations is shown in the development of the method of Clark and Lubs (1915) for the differentiation of the two main groups of the colon-aerogenes family.

Before this work on the limiting hydrogen ion concentrations in acid fermentations, Michaelis (1911) had shown that certain organisms, notably *B. typhi*, respond in much the same manner as do proteins and agglutinate within certain zones of hydrogen ion concentration with characteristic optima.

The influence of hydrogen ion concentration upon the activity of yeasts has received considerable attention of late, especially in connection with the manufacture of beer (Emslander, 1914; Lüers, 1914; Leberle and Lüers, 1914; Lüers and Adler, 1915; Hermann; R. Wahl, 1915), wine (Paul, 1914, 1915) and bread (A. Wahl, 1915; cf. Jessen Hansen, 19. 1). Its influence in yeast culture has been recently reviewed by Hägglund (1915 a and b).

The influence of hydrogen ion concentration on molds has received less attention, if we except some excellent studies of

the mold enzymes. Waterman's (1915) estimate that the critical limit for *Penicilium glaucum* is about 1×10^{-5} N. H and for *Aspergillus niger* about 4.5×10^{-5} N. H, we can not understand. It may be that he has confused Friedenthal's (1910) gram per cubic centimeter basis of hydrogen ion concentration with the generally accepted gram per liter standard. Dr. Currie of this laboratory has cultivated for us an organism which Dr. Thom has classified as an *Aspergillus niger*. When grown on a medium consisting of 1 gram KH_2PO_4, 3 grams $NaNO_3$, 0.5 gram $MgSO_4$ $7H_2O$, 100 grams sucrose, in 1000 cc. water, the medium taken from below the mycelium on the seventh day of growth had a hydrogen ion concentration of 2×10^{-2} N.

Whether different hydrogen ion concentrations have any effect upon the metabolism of molds will constitute an important part of the investigations on soft cheeses which are being carried on in this laboratory.

So numerous have become the researches demonstrating the importance of knowing or controlling the hydrogen ion concentration of biochemically active solutions that the subject is almost too large for review. The main outlines and a review of the more important literature up to 1914 will be found in the invaluable monograph by Michaelis (1914 b) or in the earlier classic papers of Sörensen (1909, 1912). We may, however, take this occasion to mention a few topics which are of special interest to the bacteriologist. Among the studies of the influence of hydrogen ion concentration upon enzyme action researches on the enzymes of yeasts and molds have occupied a prominent place. The bacterial enzymes have received less attention, although Meyer in 1911 determined the influence of hydrogen ion concentrations upon the proteases of *B. prodigiosus* and *B. pyocyaneus*. Itano (1916), in his recent paper on the relation of hydrogen ion concentration of media to the proteoclastic activity of *B. subtilis, Strept. erysipelatos* and *Strept. lacticus*, has given some further information but his references indicate that others have not given this subject the attention it deserves.

Among the enzyme studies of particular interest to bacteriologists is that of Palitzsch and Walbum (1912) on tryptic gelatin

Of almost equal importance in special fields of bacteriological research have been the numerous studies of the influence exercised by the hydrogen ions upon the physical condition of colloids and more particularly upon protein solutions. Correlated with these investigations are the studies on acid agglutination of bacteria (Michaelis 1911, Beniash 1911, and others) and their constituents (Arkwright 1914).

In the work of Jordan (1903), of Atkin (1911, 1914) and of Walbum (1915) upon various phases of haemolysis, and of Hamburger and Hekma (1908) and of Koltzoff (1914) upon phagocytosis, in the voluminous literature upon absorption phenomena, and cell permeability, and in the researches of Hildebrand (1913) and others upon inorganic solutions the influence of the activity of hydrogen ions has been revealed or actually studied in a wide variety of cases, which are of special interest to bacteriologists. Not less suggestive are the determinations of the true reaction of various natural solutions in which bacteria find their habitat.

The study of the disinfecting power of the hydrogen ions may be said to have begun with the work of Paul and Krönig (1896) and Krönig and Paul (1897) on bacteria, Clark's (1899) work with fungi and Bial's (1902) work with yeasts. These investigations, like many which were to follow, were handicapped by the lack of a method for the direct determination of hydrogen ion concentration. As late as 1906 Winslow and Lochridge, in their demonstration of the disinfecting power of hydrogen ions, had to resort to conductivity data, which are difficult to interpret when the solutions are complex. Even later, when better methods became available, elaborate researches were recorded in which the attempt was made to draw refined conclusions by comparing the effects of different acids with their dissociation constants. This procedure in other lines of investigation has yielded results which are far less certain than those obtained by direct determinations of hydrogen ion concentration, and for reasons which will shortly be mentioned. It may be well, therefore, to consider as unsettled some of the mooted points which have arisen in the study of the disinfecting power of the

hydrogen ions until some of the work which has been done even within the last year can be repeated with more modern methods of attack. It should be noted that some of the work upon this subject has indicated a not surprising specificity in the effects of different acids. Nevertheless we fail to see how these important differences can be accurately measured until a method is used which permits the determination of at least one of the products of dissociation.

Of particular interest is the work of Bruenn (1915) who found the disinfecting power of lactic and acetic acids for *B. coli* and *B. typhi* to be due to the activity of the hydrogen ions and who therefore opposed the conclusions of Paul, Birstein and Reuss (1910).

A fundamental objection which may be raised against some of the work which has been done in trying to discover the effects of acids or of the hydrogen ions is that no account has been taken of the "buffer action" in the media used. It is not a difficult matter to demonstrate theoretically and experimentally that in the presence of proper buffers acids may not furnish concentrations of the hydrogen ion which can be judged merely from an inspection of their dissociation constants. In many instances a "weak" acid may alter the hydrogen ion concentration to practically the same extent as the same quantity of a much "stronger" acid.

Unbuffered solutions, on the other hand, are susceptible to enormous changes in hydrogen ion concentration under the action of even certain of the so-called "neutral salts." Quite apart from their well-established influence upon the apparent hydrogen ion concentration of buffered solutions, such so-called neutral salts may at times exert a greater physiological influence by altering the hydrogen ion concentration of a solution than they do through the direct influence of their own ions.

These brief citations and remarks will be recognized by those who are familiar with the subject as a very inadequate sketch. They may, however, serve to indicate the basis upon which Sörensen (1912) stood when he maintained that in the study of biological processes no conclusion may be regarded as free from the possibility of error unless the influence of the hydrogen ions

has been controlled or taken into consideration. If this is true in the fields of bacteriological research, some experimental method of determining hydrogen ion concentrations which is particularly adapted to bacteriological problems is an absolute necessity.

Each of the two chief methods of determining hydrogen ion concentrations has its own merit. For accurate determinations, for the study of highly colored solutions, as a check upon colorimetric measurements and in all cases of serious importance the hydrogen electrode method should be available. It has been developed so that very high accuracy may generally be attained in less time than is required for very accurate colorimetric work. We must qualify this statement by adding; that for accurate, rapid work there is necessary temperature control, careful electrical installation, the use of a proper electrode vessel and the shaking method of establishing equilibria, attention to numerous details, and the use of a potentiometer system which is reliable.[2] Reviews of the electrometric method will be found in the monographs of Sörensen (1912) and Michaelis (1914 b). A hydrogen electrode vessel particularly adapted to accurate rapid work has been described by Clark (1915 b) and used in several bacteriological investigations. Such an equipment can now be purchased from a reliable manufacturer of electrical instruments.

There are, however, cases where the hydrogen electrode is not applicable, though we have reason to believe that these cases will become fewer as the theory and technique of electrometric measurements are perfected. There are also cases where

[2] McClendon (1915) and Bovie (1915) have described potentiometers designed to indicate directly the P_H value of a measured solution. Doubtless such instruments have special uses, but, for general work, we fail to see any real advantage in a "direct reading potentiometer." It seems almost unnecessary to call attention to the fact that, when rough measurements are made and corrections do not have to be applied, potential readings may be transformed to P_H in five seconds by means of a curve. If accurate measurements are to be made corrections must be applied, which is difficult with a direct reading potentiometer. If very rapid changes are to be followed, it is quite as easy to think of these in terms of potential as in terms of P_H. If direct readings must be had and corrections are to be neglected, a P_H scale pasted over the millivolt scale in Hildebrand's (1913) arrangement will do. We are convinced that it is well to use only a reliable universal instrument.

great accuracy is not necessary and a rapid, simple method will do. There are, furthermore, cases where it is necessary to make a great many approximate determinations of hydrogen ion concentrations, which need be of only *relatively* high accuracy. In such cases, which occur daily in the bacteriological laboratory, the colorimetric method finds its greatest usefulness.

This method has long been available, but, probably because it has not been developed to meet the particular needs of bacteriology, and also because its usefulness to the bacteriologist has not been sufficiently emphasized, it has found no extensive application in the bacteriological laboratory.

We have felt that one hindrance has been the lack of brilliant indicators which can reveal their complexion even in the presence of the coloration and the turbidity found in many culture media and cultures. This hindrance we have partially removed by assembling a series of exceptionally brilliant indicators, several of which we ourselves have synthesized for the first time. We have also studied some methods which materially aid in the examination of highly colored or turbid media, and at the same time we have developed a system of standard solutions and have simplified their preparation, so that, with regard to the several minor points which will be described, we feel that the method is readily available to the bacteriologist for routine as well as research purposes. The relative accuracy of the method will be revealed in the numerous comparisons we have made with careful electrometric determinations on a wide variety of solutions.

In order that the main outlines of the subject may not be lost in the discussion of experimental details, and in order that they may be assembled for the convenience of those bacteriologists who may desire a review of the subject, the more essential theoretical aspects will be briefly reviewed,[3] and their particular significance in bacteriological problems will be noted.

[3] An excellent elementary exposition of the theory of electrolytic dissociation is found in a little book by Talbot and Blanchard (1905). Fuller treatments will be found in any modern text book of physical chemistry. Those portions in which we are now especially interested have been treated in the monographs of Sörensen (1912) and Michaelis (1914).

Those already familiar with the subject will find the new information they desire in the following sections: A new series of standard comparison solutions is described in section V and the compositions and corresponding P_H values are given in table 1. A complete new series of indicators is listed in table 2. Comparisons of electrometric P_H determinations and colorimetric determinations made with these indicators are shown and briefly described in section XIV. A description of apparatus will be found in section XII.

SECTION II. THE METHOD OF EXPRESSING HYDROGEN ION CONCENTRATIONS

In physiological solutions there are generally encountered concentrations of the hydrogen ion which are very much smaller than the concentrations of the substances ordinarily dealt with in analytical chemistry. Nevertheless the standard of concentration remains the normal solution, which, by definition, contains the equivalent of 1 gram of hydrogen in 1 liter of the solution.[4] In these terms the hydrogen ion concentration of pure water, for instance, is 0.000,000,1 N. A convenient abbreviation of this unwieldy figure is 1×10^{-7}N. In like manner the hydrogen ion concentration of a 0.1 N NaOH solution, which is about 0.000,000,000,000,09 N, may be written 9×10^{-14}.

If the hydrogen ion concentration of pure water, which is considered to be that of *true neutrality*, is 1×10^{-7} N, it may be asked how one may speak of the hydrogen ion concentration of an *alkaline solution* and of a value as small as 9×10^{-14}. It follows from the fact that in every aqueous solution there are both hydrogen and hydroxyl ions, the relative concentrations of which are governed by the dissociation of the water itself. In the reversible reaction $H_2O \rightleftharpoons \overset{+}{H} + \overset{-}{O}H$, which expresses the dissociation of water, there is an equilibrium which may be expressed as follows:

[4] For all ordinary purposes it makes no difference in defining the normality of hydrogen ion concentrations whether we consider the atomic weight of hydrogen to be 1.0 or 1.008.

$$[H] \times [OH] = K_w$$

[H] and [OH] represent the concentrations of hydrogen and hydroxyl ions respectively, and K_w is a constant, the so-called dissociation constant of water. If K_w in the above equation is to remain a constant as [H] and [OH] change, there must be present in every aqueous solution some hydrogen ions and there must be maintained a balance between the concentrations [H] and [OH]. When by the addition of alkali, for instance, [OH] is increased there must remain sufficient hydrogen ions to make the product $[H] \times [OH]$ equal to the constant K_w.

K_w has been established by various methods with remarkable agreement. Although it varies with the temperature it may be considered here to be equal to 1×10^{-14}. Assuming, as we must, that in perfectly pure water the concentrations of the hydrogen and hydroxyl ions are equal we have:

$$[H]^2 = [OH]^2 = 1 \times 10^{-14}$$

Hence:— $$[H] = [OH] = 1 \times 10^{-7}$$

This concentration of hydrogen or hydroxyl ions (1×10^{-7}) is called *the true neutral point.* Solutions with hydrogen ion concentrations greater than or hydroxyl ion concentrations less than 1×10^{-7} are called acid solutions. Those with hydrogen ion concentrations less than or hydroxyl ion concentrations greater than 1×10^{-7} are called alkaline solutions. Since $[OH] = \frac{K_w}{[H]}$ the hydroxyl ion concentration of a solution may be readily calculated if its hydrogen ion concentration is known, or *vice versa.* As a matter of practice it is more convenient to determine hydrogen ion concentrations, and as a matter of uniformity only the hydrogen ion concentration of a solution is generally mentioned as was suggested by Friedenthal (1904).

It is often necessary to plot hydrogen ion concentrations upon coördinate paper. In this case a difficulty arises when one attempts to lay off on a chart concentrations which are widely different. If one wishes, for instance to show a difference between 1×10^{-8} and 3×10^{-8} the chart would not be within bounds when one came to the magnitudes 1×10^{-4} and 3×10^{-4}. We could, however, plot these values logarithmically as Hender-

son (1908) did in plotting dissociation curves. It has been very generally agreed that the logarithm of the reciprocal of the hydrogen ion concentration should be used. To this Sörensen (1909 a and b) has given the symbol P_H^+ which we shall write P_H. This particular function of the hydrogen ion concentration is especially convenient for the following reason among others. In the determination of hydrogen ion concentrations by the electrometric method the potentials measured are introduced into an equation which contains the term log. $\frac{1}{[H]}$. The value of this term is obtained very directly and serves to characterize a hydrogen ion concentration quite as well as if the calculation were carried further to obtain the actual value of [H].

The relation of P_H to [H] may be shown by the following examples. Since $P_H = \log. \frac{1}{[H]}$,

$$\text{if } [H] = 2 \times 10^{-4},\ P_H = \log. \frac{1}{2 \times 10^{-4}} = \log.\ 5{,}000 = 3.699$$

and

$$\text{if } [H] = 4 \times 10^{-6},\ P_H = \log. \frac{1}{4 \times 10^{-6}} = \log.\ 250{,}000 = 5.398$$

The following section of a table showing the relation of P_H to [H] will illustrate the construction of the whole.

P_H	[H]
5.0	1.0×10^{-5}
5.1	7.9×10^{-6}
5.2	6.3×10^{-6}
5.3	5.0×10^{-6}
5.4	4.0×10^{-6}
5.5	3.2×10^{-6}
5.6	2.5×10^{-6}
5.7	2.0×10^{-6}
5.8	1.6×10^{-6}
5.9	1.2×10^{-6}
6.0	1.0×10^{-6}
6.1	7.9×10^{-7}
6.2	6.3×10^{-7}

—etc.—

The use of Sörensen's P_H has met with superficial objections such as those set up by Armstrong and Armstrong (1913), and other scales of reaction have been proposed such as the very inadequate one suggested by Walker and Kay (1912). Nevertheless P_H will doubtless continue to be used, not only as a matter of custom but because it is a fundamentally logical unit. Our methods of determining hydrogen ion concentrations have errors which are more or less directly proportional to some constant difference of P_H but are not directly proportional to any constant difference in [H]. Differences in P_H rather than differences in [H] are of physiological importance, and finally the graphic treatment of the relation of hydrogen ion concentrations to the dissociation of acids and bases may be expressed most beautifully when P_H values rather than the corresponding [H] values are used as Henderson and Sörensen and others have shown. We shall therefore use the P_H values rather than the corresponding hydrogen ion concentrations, in the following discussions.

It should be particularly noted that as P_H increases [H] decreases and *vice versa*. In plotting curves where we wish to show the relation between P_H and some other quantity, such as the amount of acid added to a medium, we shall depart from the common practice of showing a rise in P_H by a rise in the curve, instead plotting P_H so that a rise in the curve at once indicates to the eye a rise in acidity.

SECTION III. BUFFER ACTION

If we were to add to 1 liter of perfectly pure water of $P_H = 7.0$, 1 cc. of 0.01 N HCl, the resulting solution would be about $P_H = 5.0$ and very toxic to many bacteria. If, on the other hand, we were to add this same amount of acid to a liter of a standard beef infusion medium of $P_H = 7.0$, the resulting change in P_H would be hardly appreciable. This power of certain solutions to resist change in reaction was commented upon by Fernbach and Hubert (1900) who likened the resistance of phosphate solutions to a "tampon." The word was adopted by Sörensen (1909 a) and in the German rendition of his paper (1909 b) it

became "puffer" and thence the English "buffer." There has been some objection to this word so applied, but it has now acquired a clear technical meaning and is so generally used that it should probably be retained. By buffer action then we mean the ability of a solution to resist change in P_H through the addition or loss of acid or alkali. This may be illustrated by titration curves such as those shown in figures 1, 2 and 3. The construction of such curves may be illustrated by the following example.

A 1 per cent solution of Witte peptone was found to have a P_H value of 6.87. To equal portions of the solution were added successively increasing amounts of 0.1 N lactic acid and the resulting P_H was measured in each case. There were also added to equal portions of the solution successively increasing amounts of 0.1 N NaOH and the resulting P_H was measured in each case. The P_H values were then plotted on cross section paper as ordinates against the amount of acid or alkali added in each case as abscissas. This gave the curve shown in figure 1. The other curve shown in this figure was constructed with data obtained with a 5 per cent solution of Witte peptone. The curves of figures 2 and 3 were obtained in a similar way.

These curves illustrate the following points. Figure 1 shows that the buffer action of a solution is dependent upon the concentration of the constituents. The 5 per cent solution is much more resistant to change in P_H than the 1 per cent. solution. It will also be noticed that in either case the buffer action is not the same at all points in the curve. In other words the buffer action can not be expressed by a constant but must be determined for each region of P_H. This is illustrated even more clearly by the titration curve for phosphoric acid, (fig. 2). At the point where the solution contains only the primary phosphate, and again where it contains only the secondary phosphate there is very little buffer effect indeed. Furthermore the buffer action of a solution may not be due entirely to the nature of the constituents titrated but also to the nature of the substance with which it is titrated. This point may be illustrated by titrating a beef infusion medium in the one case with hydro-

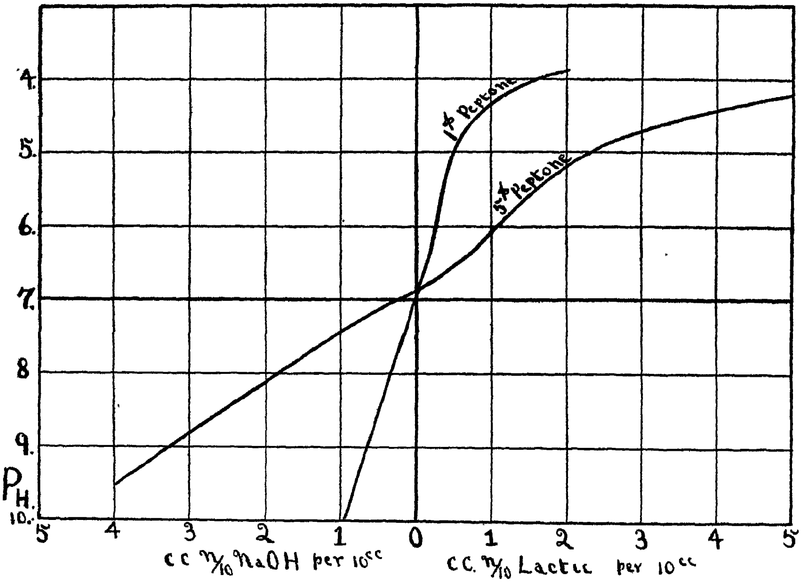

Fig. 1. Titration Curves of Peptone Solutions

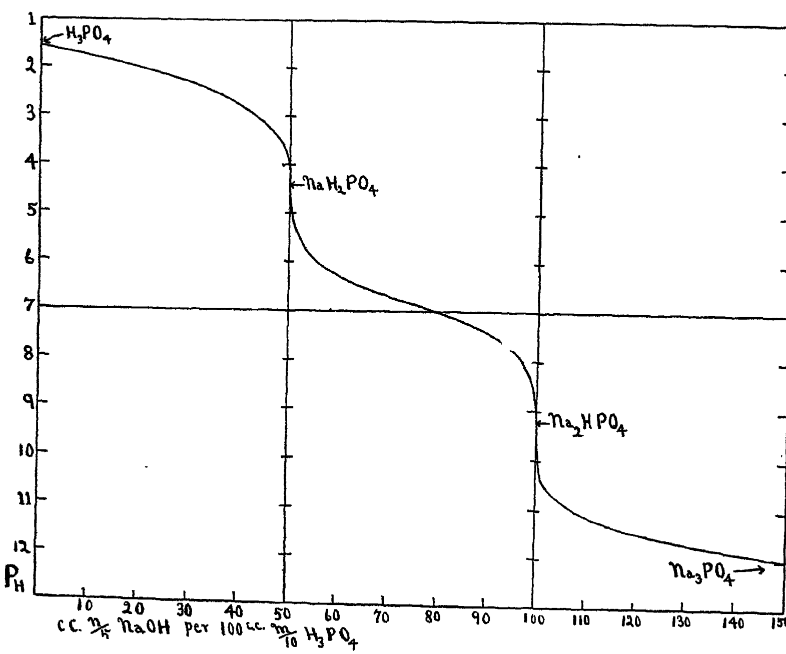

Fig. 2. Titration Curve of Ortho Phosphoric Acid

chloric acid and in the other case with acetic acid both of the same normality (see fig. 3). It will be seen that at first the two curves are identical. As the region is approached where the dissociation of the "weak" acetic acid is itself suppressed because of the accumulation of acetate ions and the high concentration of the hydrogen ions, further addition of this acid has com-

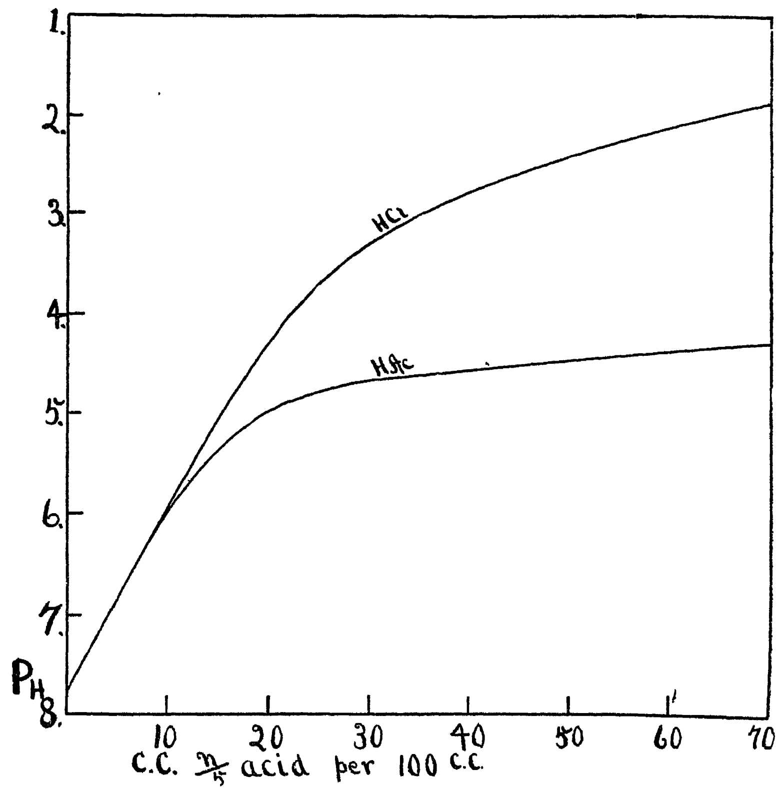

FIG. 3. TITRATION CURVES OF A BEEF INFUSION MEDIUM

paratively little effect. The strongly dissociated hydrochloric acid, on the other hand continues to be effective until it too, at very high hydrogen ion concentrations, is suppressed.

These examples will suffice to make it evident that the buffer action of a solution is dependent upon the nature and the concentrations of the constituents, upon the P_H region where the

buffer action is measured, and upon the nature of the acid or alkali added. To connect all these variables is a difficult problem. Koppel and Spiro (1914) have attempted to do so, but they have necessarily had to leave out of consideration another factor. If there are present any bodies which tend to absorb any of the constituents of a solution which can affect the hydrogen ion concentration of a solution, these bodies will tend to act as buffers, or will affect the buffer action of the solution. Henderson (1909) has called attention to this and Bovie (1915) has shown in a very interesting way the buffer action of charcoal. Since some culture media or cultures, and many of the solutions whose buffer action must be studied for physiological purposes, contain undissolved or colloidal material which may act in this way, it seems best to consider buffer action in its broadest sense, and to express it by the relative slopes of titration curves determined experimentally. Further illustrations of titration curves of culture media will be found in the papers of Clark (1915b and e) and of Bovie (1915). Titration curves of some inorganic solutions will be found in a paper by Hildebrand (1913). More theoretical treatments of the subject are given in the papers of Henderson (1909), Sörensen (1909 a and b), Sörensen (1912,) Michaelis (1914 b) and Koppel and Spiro (1914).

The standard comparison solutions used in the colorimetric determination of P_H are buffer solutions. Only those solutions may be used for standards which have a buffer action sufficient to resist the effect of unavoidable slight contamination. The curves for some standard solutions are shown in figure 4.

SECTION IV. OUTLINE OF THE COLORIMETRIC METHOD

The colorimetric method of determining hydrogen ion concentrations is based upon the fact that each indicator has a characteristic zone of hydrogen ion concentrations within which its color changes occur. Thus phenolphthalein is colorless above $[H] = 1 \times 10^{-8}$ ($P_H = 8$), but, if the solution is slightly more alkaline the pink color appears and becomes more intense with increased alkalinity until the indicator seems to have its full

color at about $P_H = 10$. Methyl red, which is yellow in alkaline, neutral and slightly acid solutions, and red in very acid solutions, exhibits its intermediate colors between $P_H = 4$ and $P_H = 6$.

If certain physical constants of indicators were well enough established, and, if we had convenient means of accurately and rapidly determining the percentage color change induced, we could use such data to determine hydrogen ion concentrations; and such a method could be made in large measure independent of all others.

It is much more convenient however to have a set of buffer solutions whose P_H values have been accurately defined by hydrogen electrode measurements, and to compare their color after the addition of the proper indicator with the color of the tested solution to which a like quantity of the indicator has been added.

The essentials then are: first a set of indicators to cover the ranges of P_H to be studied, and second a set of standard buffer solutions which may be used within these ranges.

SECTION V. STANDARD SOLUTIONS

The standard solutions used in the colorimetric method of determining hydrogen ion concentrations are buffer solutions with such well defined compositions that they can be accurately reproduced, and with P_H values accurately defined by hydrogen electrode measurements. They generally consist of mixtures of some acid and its alkali salt. Several such mixtures have been carefully studied. An excellent set has been described by Sörensen (1912). This set may be supplemented by the acetic acid—sodium acetate mixtures, most careful measurements of which have been made by Walpole (1914 b), and by Palitzsch's (1915) excellent boric acid-borax mixtures. In a recent paper (Clark and Lubs, 1916) we have described a set of standards which we believe is well designed for ease, convenience and accuracy of preparation. We called attention to certain slight difficulties in the preparation of the older standards, which we believe

should be avoided if the colorimetric method is to come into the wide routine use which we hope it will enjoy in bacteriological studies; and we noted the advantages of the following set of mixtures:

Potassium chlorid + HCl
Acid potassium phthalate + HCl
Acid potassium phthalate + NaOH
Acid potassium phosphate + NaOH
Boric acid, KCl + NaOH

For a discussion of these mixtures, the methods used in determining their P_H values, and the potential measurements we refer the reader to the original paper [Journal of Biological Chemistry, 1916, vol. xxv, no. 3, p. 479]. We may proceed at once to describe the details of preparation.

The various mixtures are made up from the following stock solutions: M/5 potassium chlorid (KCl), M/5 acid potassium phosphate (KH_2PO_4), M/5 acid potassium phthalate ($KHC_8H_4O_4$), M/5 boric acid with M/5 potassium chlorid (H_3BO_3, KCl), M/5 sodium hydroxid (NaOH), and M/5 hydrochloric acid (HCl). Although the subsequent mixtures are diluted to M/20 the above concentrations of the stock solutions are convenient for several reasons.

The water used in the crystallization of the salts and in the preparation of the stock solutions and mixtures should be redistilled. So-called "conductivity water," which is distilled first from acid chromate solution and again from barium hydroxid, is recommended.

M/5 potassium chlorid solution. (This solution will not be necessary except in the preparation of the most acid series of mixtures.) The salt should be recrystallized three or four times and dried in an oven at about 120°C. for two days. The fifth molecular solution contains 14.912 grams in 1 liter.

M/5 Acid potassium phthalate solution. Acid potassium phthalate may be prepared by the method of Dodge (1915) modified as follows. Make up a concentrated potassium hydroxid solution by dissolving about 60 grams of a high grade sample in about 400 cc. of water. To this add 50 grams of the commercial

resublimed anhydrid of ortho phthalic acid. Test a cool portion of the solution with phenol phthalein. If the solution is still alkaline, add more phthalic anhydrid; if acid, add more KOH. When roughly adjusted to a slight pink with phenol phthalein[5] add as much more phthalic anhydrid as the solution contains and heat till all is dissolved. Filter while hot, and allow the crystallization to take place slowly. The crystals should be drained with suction and recrystallized at least twice from distilled water.[6] Dry the salt at 110°–115°C. to constant weight.

A fifth molecular solution contains 40.828 grams of the salt in 1 liter of the solution.

M/5 acid potassium phosphate solution. A high grade commercial sample of the salt is recrystallized at least three times from distilled water and dried to constant weight at 110°–115°C. A fifth molecular solution should contain in 1 liter 27.232 grams. The solution should be distinctly red with methyl red and distinctly blue with brom phenol blue.

M/5 boric acid M/5 potassium chloride. Boric acid should be recrystallized several times from distilled water. It should be air dried[7] in thin layers between filter paper and the constancy of weight established by drying small samples in thin layers in a desiccator over $CaCl_2$. Purification of KCl has already been noted. It is added to the boric acid solution to bring the salt concentration in the borate mixtures to a point comparable with that of the phosphate mixtures so that colorimetric checks may be obtained with the two series where they overlap. One liter of the solution should contain 12.4048 grams of boric acid and 14.912 grams of potassium chlorid.

M/5 Sodium hydroxid solution. This solution is the most difficult to prepare, since it should be as free as possible from carbonate. A solution of sufficient purity for the present purposes may be prepared from a high grade sample of the hydroxid

[5] Use a diluted portion for the final test.

[6] While the present war-price of phthalic acid continues it will be well to recover the phthalic acid from the mother liquors by acidifying these. The recovered phthalic acid may be easily and economically purified by several recrystallizations.

[7] Boric acid begins to lose "water of constitution" above 50°C.

in the following manner. Dissolve 100 grams NaOH in 100 cc. distilled water in a Jena or Pyrex glass Erlenmeyer flask. Cover the mouth of the flask with tin foil and allow the solution to stand over night till the carbonate has mostly settled. Then prepare a filter as follows. Cut a "hardened" filter paper to fit a Buchner funnel. Treat it with warm strong [1 : 1] NaOH solution. After a few minutes decant the sodium hydroxid and wash the paper first with absolute alcohol, then with dilute alcohol, and finally with large quantities of distilled water. Place the paper on the Buchner funnel and apply gentle suction until the greater part of the water has evaporated but do not dry so that the paper curls. Now pour the concentrated alkali upon the middle of the paper, spread it with a glass rod making sure that the paper, under gentle suction, adheres well to the funnel, and draw the solution through with suction. The clear filtrate is now diluted quickly, after rough calculation, to a solution somewhat more concentrated than N/1. Withdraw 10 cc. of this dilution and standardize roughly with an acid solution of known strength, or with a sample of acid potassium phthalate. From this approximate standardization calculate the dilution required to furnish an M/5 solution. Make the required dilution with the least possible exposure, and pour the solution into a *paraffined* bottle to which a calibrated 50 cc. burette and soda-lime guard tubes have been attached. See section XII. The solution should now be most carefully standardized. One of the simplest methods of doing this, and one which should always be used in this instance, is the method of Dodge (1915) in which use is made of the acid potassium phthalate purified as already described. Weigh out accurately on a chemical balance with standardized weights several portions of the salt of about 1.6 grams each. Dissolve in about 20 cc. distilled water and add 4 drops phenol phthalein. Pass a stream of CO_2—free air through the solution and titrate with the alkali till a faint but distinct and permanent pink is developed. It is preferable to use a factor with the solution rather than attempt adjustment to an exact M/5 solution.

M/5 Hydrochloric acid solution. Dilute a high grade of hydro-

chloric acid solution to about 20 per cent and distill. Dilute the distillate to approximately M/5 and standardize with the sodium hydroxid solution previously described. If convenient, it is well to standardize this solution carefully by the silver chlorid method and check with the standardized alkali.

The only solution which it is absolutely necessary to protect from the CO_2 of the atmosphere is the sodium hydroxid solution. Therefore all but this solution may be stored in ordinary bottles of resistant glass. The salt solutions, if adjusted to exactly M/5, may be measured from clean calibrated pipettes.

These constitute the stock solutions from which the mixtures are prepared. The general relationships of these mixtures to their P_H values are shown in figure 4. In this figure P_H values are plotted as ordinates against X cc. of acid or alkali as abscissae. It will be found convenient to plot this figure from the following table with greatly enlarged scale so that it may be used as is Sörensen's chart (1909 a). The composition of the mixtures at even intervals of 0.2 P_H are given in table 1.

In any measurement the apportionment of scale divisions should accord with the precision. Scale divisions should not be so coarse that interpolations tax the judgment nor so fine as to be ridiculous. What scale divisions are best in the method under discussion it is difficult to decide, since the precision which may be attained depends somewhat upon the ability of the individual eye, and upon the material examined, as well as upon the means and the judgment used in overcoming certain difficulties which we shall mention later. Certain general considerations have led us to believe that for most bacteriological work estimation of P_H values to the nearest 0.1 division is sufficiently precise, and that this precision can be obtained when the composition of the medium permits if the comparison standards differ by increments of 0.2 P_H. Sörensen (1909 a and b) has arranged the standard solutions to differ by even parts of the components, a system which furnishes uneven increments in P_H. Michaelis, (1910) on the other hand, makes his standards vary by about 0.3 P_H so that the corresponding hydrogen ion

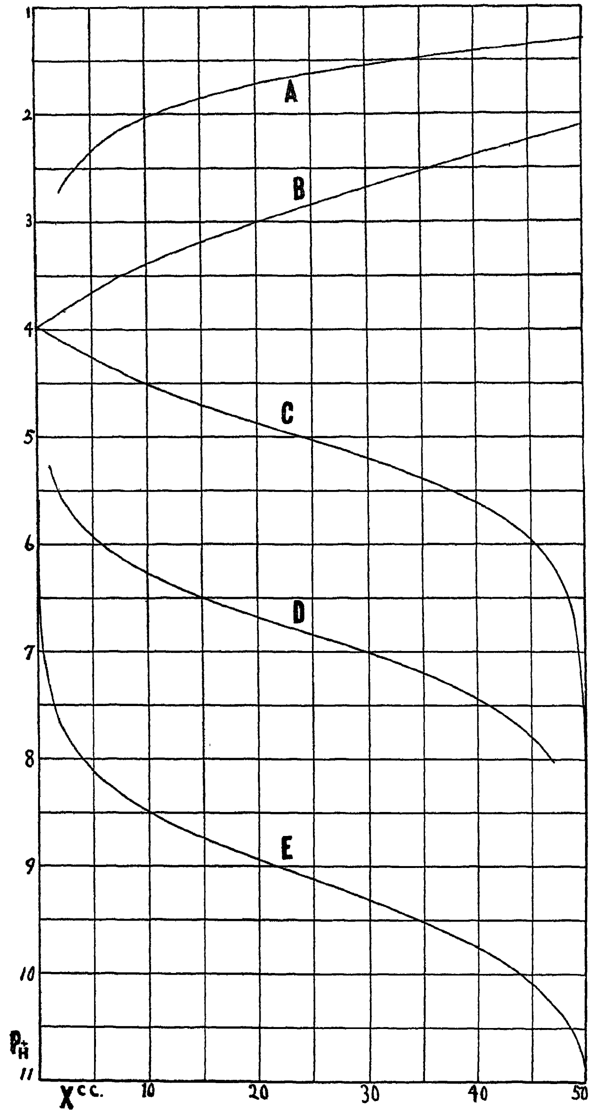

FIG. 4. P_H VALUES OF THE MIXTURES:

A.	50 cc. 0.2 M KCl	+ X cc. 0.2 M HCl	Diluted to 200
B.	50 cc. 0.2 M HKPhthalate	+ X cc. 0.2 M HCl	Diluted to 200
C.	50 cc. 0.2 M HKPhthalate	+ X cc. 0.2 M NaOH	Diluted to 200
D.	50 cc. 0.2 M H_2KPO_4	+ X cc. 0.2 M NaOH	Diluted to 200
E.	50 cc. 0.2 M H_3BO_3, 0.2 M KCl	+ X cc. 0.2 M NaOH	Diluted to 200

concentrations are approximately doubled at each step. Our experience has convinced us of the advantage of the 0.2 P_H increments we are recommending.

We have found it convenient to prepare 200 cc. of each of the mixtures and to preserve them in bottles each of which has its own 10 cc. pipette thrust through the stopper. It takes but little more time to prepare 200 cc. than it does to prepare a 10

TABLE 1

Compositions of mixtures giving P_H *values at 20°C. at intervals of 0.2*

KCl-HCl mixtures

P_H	Composition		
1.0	50 cc. M/5 KCl	97.0 cc. M/5 HCl	Dilute to 200 cc.
1.2	50 cc. M/5 KCl	64.5 cc. M/5 HCl	Dilute to 200 cc.
1.4	50 cc. M/5 KCl	41.5 cc. M/5 HCl	Dilute to 200 cc.
1.6	50 cc. M/5 KCl	26.3 cc. M/5 HCl	Dilute to 200 cc.
1.8	50 cc. M/5 KCl	16.6 cc. M/5 HCl	Dilute to 200 cc.
2.0	50 cc. M/5 KCl	10.6 cc. M/5 HCl	Dilute to 200 cc.
2.2	50 cc. M/5 KCl	6.7 cc. M/5 HCl	Dilute to 200 cc.

Phthalate-HCl mixtures

2.2	50 cc. M/5 KHPhthalate	46.70 cc. M/5 HCl	Dilute to 200 cc.
2.4	50 cc. M/5 KHPhthalate	39.60 cc. M/5 HCl	Dilute to 200 cc.
2.6	50 cc. M/5 KHPhthalate	32.95 cc. M/5 HCl	Dilute to 200 cc.
2.8	50 cc. M/5 KHPhthalate	26.42 cc. M/5 HCl	Dilute to 200 cc.
3.0	50 cc. M/5 KHPhthalate	20.32 cc. M/5 HCl	Dilute to 200 cc.
3.2	50 cc. M/5 KHPhthalate	14.70 cc. M/5 HCl	Dilute to 200 cc.
3.4	50 cc. M/5 KHPhthalate	9.90 cc. M/5 HCl	Dilute to 200 cc.
3.6	50 cc. M/5 KHPhthalate	5.97 cc. M/5 HCl	Dilute to 200 cc.
3.8	50 cc. M/5 KHPhthalate	2.63 cc. M/5 HCl	Dilute to 200 cc.

Phthalate-NaOH mixtures

4.0	50 cc. M/5 KHPhthalate	0.40 cc. M/5 NaOH	Dilute to 200 cc.
4.2	50 cc. M/5 KHPhthalate	3.70 cc. M/5 NaOH	Dilute to 200 cc.
4.4	50 cc. M/5 KHPhthalate	7.50 cc. M/5 NaOH	Dilute to 200 cc.
4.6	50 cc. M/5 KHPhthalate	12.15 cc. M/5 NaOH	Dilute to 200 cc.
4.8	50 cc. M/5 KHPhthalate	17.70 cc. M/5 NaOH	Dilute to 200 cc.
5.0	50 cc. M/5 KHPhthalate	23.85 cc. M/5 NaOH	Dilute to 200 cc.
5.2	50 cc. M/5 KHPhthalate	29.95 cc. M/5 NaOH	Dilute to 200 cc.
5.4	50 cc. M/5 KHPhthalate	35.45 cc. M/5 NaOH	Dilute to 200 cc.
5.6	50 cc. M/5 KHPhthalate	39.85 cc. M/5 NaOH	Dilute to 200 cc.
5.8	50 cc. M/5 KHPhthalate	43.00 cc. M/5 NaOH	Dilute to 200 cc.
6.0	50 cc. M/5 KHPhthalate	45.45 cc. M/5 NaOH	Dilute to 200 cc.
6.2	50 cc. M/5 KHPhthalate	47.00 cc. M/5 NaOH	Dilute to 200 cc.

TABLE 1—*Continued*

KH_2PO_4-NaOH mixtures

5.8	50 cc. M/5 KH_2PO_4	3.72 cc. M/5 NaOH	Dilute to 200 cc.
6.0	50 cc. M/5 KH_2PO_4	5.70 cc. M/5 NaOH	Dilute to 200 cc.
6.2	50 cc. M/5 KH_2PO_4	8.60 cc. M/5 NaOH	Dilute to 200 cc.
6.4	50 cc. M/5 KH_2PO_4	12.60 cc. M/5 NaOH	Dilute to 200 cc.
6.6	50 cc. M/5 KH_2PO_4	17.80 cc. M/5 NaOH	Dilute to 200 cc.
6.8	50 cc. M/5 KH_2PO_4	23.65 cc. M/5 NaOH	Dilute to 200 cc.
7.0	50 cc. M/5 KH_2PO_4	29.63 cc. M/5 NaOH	Dilute to 200 cc.
7.2	50 cc. M/5 KH_2PO_4	35.00 cc. M/5 NaOH	Dilute to 200 cc.
7.4	50 cc. M/5 KH_2PO_4	39.50 cc. M/5 NaOH	Dilute to 200 cc.
7.6	50 cc. M/5 KH_2PO_4	42.80 cc. M/5 NaOH	Dilute to 200 cc.
7.8	50 cc. M/5 KH_2PO_4	45.20 cc. M/5 NaOH	Dilute to 200 cc.
8.0	50 cc. M/5 KH_2PO_4	46.80 cc. M/5 NaOH	Dilute to 200 cc.

Boric acid, KCl-NaOH mixtures

7.8	50 cc. M/5 H_3BO_3, M/5 KCl	2.61 cc. M/5 NaOH	Dilute to 200 cc.
8.0	50 cc. M/5 H_3BO_3, M/5 KCl	3.97 cc. M/5 NaOH	Dilute to 200 cc.
8.2	50 cc. M/5 H_3BO_3, M/5 KCl	5.90 cc. M/5 NaOH	Dilute to 200 cc.
8.4	50 cc. M/5 H_3BO_3, M/5 KCl	8.50 cc. M/5 NaOH	Dilute to 200 cc.
8.6	50 cc. M/5 H_3BO_3, M/5 KCl	12.00 cc. M/5 NaOH	Dilute to 200 cc.
8.8	50 cc. M/5 H_3BO_3, M/5 KCl	16.30 cc. M/5 NaOH	Dilute to 200 cc.
9.0	50 cc. M/5 H_3BO_3, M/5 KCl	21.30 cc. M/5 NaOH	Dilute to 200 cc.
9.2	50 cc. M/5 H_3BO_3, M/5 KCl	26.70 cc. M/5 NaOH	Dilute to 200 cc.
9.4	50 cc. M/5 H_3BO_3, M/5 KCl	32.00 cc. M/5 NaOH	Dilute to 200 cc.
9.6	50 cc. M/5 H_3BO_3, M/5 KCl	36.85 cc. M/5 NaOH	Dilute to 200 cc.
9.8	50 cc. M/5 H_3BO_3, M/5 KCl	40.80 cc. M/5 NaOH	Dilute to 200 cc.
10.0	50 cc. M/5 H_3BO_3, M/5 KCl	43.90 cc. M/5 NaOH	Dilute to 200 cc.

cc. portion, and if the larger volume is prepared there will not only be a sufficient quantity for a day's work but there will be some on hand for the occasional test.

Unless electrometric measurements can be used as control, we urge the most scrupulous care in the preparation and preservation of the standards. Mixtures over a week old should be discarded. We believe that the use of preservatives, such as toluol, is likely to lead to a false sense of security and we have therefore not attempted to investigate their use. We have specified several recrystallizations of the salts used because no commercial samples which we have yet encountered are reliable. Dependence on these commercial salts has caused errors in the preparation of the old standards in important cases.

It is important to check the consistency of any particular set of these mixtures by comparing "5.8" and "6.2 phthalate" with "5.8" and "6.2 phosphate" using brom cresol purple. Also "7.8" and "8.0 phosphate" should be compared with the corresponding borates using cresol red.

SECTION VI. CHOICE OF INDICATORS

There is an almost unlimited number of compounds which have indicator properties. Among those of plant origin litmus and alizarine and among those of animal origin cochineal are the most familiar. An enormous number of plant pigments have been described, and the indicator properties of several have been noted. These have generally been described merely as indicators with no data of any value. Noteworthy exceptions have been Walpole's (1913) treatment of litmus and Walbum's (1913 a) study of the coloring principle of the red cabbage. As Walbum (1913 b) has pointed out, the mere description of a substance as an indicator is of no value whatever for biochemical purposes until its conduct in the presence of the substances found in biochemical solutions has been studied.

The indicators of natural origin deserve more study not only because of their possible general usefulness but because in certain instances, if they can be shown to exist in true solution, they may be made to furnish indications of the true reactions of the plasma in which they occur. They will then be useful in studies such as those attempted by Harvey (1913, 1915) and by Crozier (1916) upon the penetration of cells by acids and bases. In other instances their indications of the true reaction of plant tissues or juices may be of direct value to the plant pathologist.[8] Some of the pigments of bacterial origin are also worthy of investigation from the indicator point of view. Aside from the intrinsic interest of such studies they might reveal the possi-

[8] Some abortive attempts to connect the immunity of certain plant tissues with their "titratable acidities" might have been fruitful had the hydrogen ion concentrations been studied. Wagner's (1915) study of the hydrogen ion concentrations of infected plant "cell sap" seems to be a step in this direction.

bility of distinguishing these pigments by their characteristic transformation points.

Litmus and azolitmin have rendered valuable service in the survey work of bacteriology and may be given special notice. Ordinary litmus is a complex (Glazer, 1901), the composition and sensitivity of which will vary with the nine or more different methods of purification and with the source. The azolitmin of commerce, which is probably a constituent of litmus (Glazer), is of uncertain composition (Scheitz, 1910). It is very generally recognized that both litmus and azolitmin are inferior indicators which merit mention in only a few of the modern treatises.

With the development of organic chemistry many of the plant pigments have become products of the laboratory, and among the vast number of new compounds which have been synthesized are many which have admirable indicator properties. These synthetic indicators have for the most part displaced the natural indicators so that litmus and alizarine, turmeric and cochineal are relatively unfamiliar substances in the modern chemical laboratory. Indeed Bjerrum (1915) has stated that for most titrimetric purposes the two synthetic indicators methyl red and phenol phthalein are all that are necessary.

On the other hand, several indicators are required for the colorimetric determination of hydrogen ion concentrations. After Salessky (1904) in Nernst's laboratory had determined the hydrogen ion concentrations at which several indicators change, Friedenthal, and Fels, and Salm at about the same time (1904) placed the use of indicators upon a more comprehensive basis. It remained however for Sörensen (1909 a and b) not only to make the method practical and accurate in many details, but to reject the less useful indicators and to furnish a reliable selection. Indeed not the least of Sörensen's contributions has been the laborious elimination of inferior indicators. We need now no longer be discouraged by the bewildering choice presented in the elaborate indicator charts such as the classic one of Salm (1906) or the later one of Thiel (1911). Emphasis may be placed upon this part of Sörensen's work because the inferior indicators are still being used by many

unaware of their defects. We are surprised to find the reliance which Crile (1915) and Crozier, Rogers and Harrison (1915) have placed upon alizarine sulfonate. Walpole (1914 a) says "this indicator merits no place in any list of approved indicators for H concentration measurement." This is a strong statement which we believe the Cleveland authors should meet by more data than they have given, especially when we consider the profound importance of the phenomena which they have studied with this indicator.

Sörensen's selection was based largely upon the protein and salt errors which limit the usefulness of practically every indicator when applied to biological fluids. Those indicators chosen were characterized by *relatively* small susceptibility to the influence of protein and salts. To this list have since been added several other useful indicators. α-naphthol phthalein, first made by Grabowski (1871), was shown to be valuable by the studies of Sörensen and Palitzsch (1910). Methyl red [Rupp and Loose 1908] was given a permanent place by the investigations of Palitzsch (1911 b). Some indication of the usefulness of phenol sulfon phthalein [Sohon 1898] was suggested by the work of Levy, Rowntree and Marriot (1915). Several other indicators such as 2–5 di nitro hydro quinone, studied by Henderson and Forbes (1910), and the several indicators which were reviewed by Walpole (1914 a) may find uses. Hottinger (1914) has described an indicator which he isolated from lacmoid and which he calls "lacmosol," and Scatchard and Bogert (1916) have suggested that di nitro benzoylene urea is useful in biological solutions.

To these indicators which have been studied to determine their usefulness in the determination of hydrogen ion concentrations may be added the ever increasing list of indicators which have been described merely as indicators but whose usefulness remains to be determined. Dox's (1915) phenol quinolinein, the several indicators of the methyl red series which Rupp (1915) has again taken up, numerous derivatives of phenol phthalein, as well as many other indicators which are men-

tioned both in the old literature and the new, await a study of their usefulness.

Our own starting point was the selection of Sörensen (1909 a and b) supplemented by methyl red, α-napthol phthalein and phenol sulfon phthalein. Several of the Sörensen indicators were not available. We experimented with Henderson and Forbes' di nitro hydro quinone, Hottinger's lacmosol, several of the older synthetic indicators, Walbum's red cabbage extract, Sacher's (1910) red radish extract, Barthe's (1913) red potato extract, and several other natural indicators. Nierenstein (see Walpole) kindly sent us a sample of his indicator but we have been unable to make a careful study of it as yet.

The brilliancy of methyl red and of phenol sulfon phthalein led us to study other indicators of these two distinct types. Our preliminary report [Lubs and Clark 1915] will be found elsewhere.

In the methyl red series we studied the following:

o-carboxy benzene azo mono methyl aniline, Sive and Jones (1915),

o-carboxy benzene azo di methyl aniline, Rupp and Loose (1908).

o-carboxy benzene azo mono ethyl aniline, Lubs and Clark (1915).

o-carboxy benzene azo di ethyl aniline, Lubs and Clark (1915).

o-carboxy benzene azo mono propyl aniline, Lubs and Clark (1915).

o-carboxy benzene azo di propyl aniline, Lubs and Clark (1915).

o-carboxy benzene azo (?) amyl aniline[9] Lubs and Clark (1915).

[9] This is probably the mono alkyl compound. It was prepared by coupling di iso amyl aniline with diazotized anthranilic acid. By analogy with the preparation of di methyl red for instance, the di alkyl indicator should have been obtained. Karrer (1915), however, has shown that in the coupling of di butyl or di amyl aniline with a diazotized compound one alkyl group is split off. The weak color of the compound which we obtained indicates, by analogy with the weak color of the mono methyl, mono ethyl and mono propyl homologues, that it is the mono amyl compound instead of the di amyl compound. Limitations in the amount of materials available prevented adequate analysis to determine this.

o-carboxy benzene azo di methyl α naphthyl amine, Howard and Pope (1911).

o-carboxy benzene azo α naphthyl amine, Howard and Pope (1911).

o-carboxy benzene azo di phenyl amine, Howard and Pope (1911).

Meta carboxy benzene azo di methyl aniline[10] Lubs and Clark.

The mono alkyl homologues of methyl red were found to be much less brilliant than the di alkyl compounds and were therefore rejected. For the same reason or because of large protein errors we rejected the other compounds with the exception of di ethyl and di propyl red. Of these we have retained di propyl red because it is very useful in solutions of a little lower hydrogen ion concentration than those which may be studied with methyl red. Di propyl red which we shall henceforth refer to as propyl red, has some disadvantage because of its slight solubility, but this does not seriously interfere with its usefulness. Methyl red, o-carboxy benzene azo di methyl aniline, has already been extensively studied by others.

An indicator of the phthalein series which we should have mentioned in our first compilation is ortho cresol phthalein, first made by Fraude (1880). Its color change occurs in about the same range as that of phenol phthalein and is so much more brilliant that we believe ortho cresol phthalein can with advantage replace phenol phthalein for titrations.

Levy, Rowntree and Marriot first called attention to the usefulness of phenol sulfon phthalein for the determination of the hydrogen ion concentrations of dialized blood serum. This indicator, first made by Sohon (1898) in Remsen's laboratory, has received considerable attention from Acree and his students (see White 1915 and White and Acree, 1915) because it furnishes excellent material for the study of the quinone-phenolate theory of color change. New derivatives of phenol sulfon phthalein have been synthesized by Acree and his students for the purpose

[10] This indicator was prepared since the publication of our first report. It was prepared by coupling di methyl aniline with diazotized meta amino benzoic acid.

of furthering studies on tautomerism and the theory of color change in indicators, and new homologues and their derivatives have been synthesized by us in order to obtain satisfactory indicators for the determination of hydrogen ion concentration. Our preliminary work with these compounds has been briefly described in a former paper [Lubs and Clark 1915]. In this paper will be found those essential electrometric comparisons which are necessary before the usefulness of any indicator in biological solutions is established and which were neglected by Levy, Rowntree and Marriot (1915) and by Hurwitz, Meyer and Ostenberg (1915, 1916) in their studies of phenol sulfon phthalein.

In the sulfon phthalein series we studied the following:

Phenol sulfon phthalein, Sohon (1898).

Tetra nitro phenol sulfon phthalein, White and Acree (1915).

Phenol nitro sulfon phthalein, Lubs and Clark (1915).

Tetra bromo phenol sulfon phthalein, White and Acree (1915).

Tetra chloro phenol sulfon phthalein,[11] Lubs and Clark.

Ortho cresol sulfon phthalein, Sohon (1898).

Di bromo ortho cresol sulfon phthalein, Sohon (1898).

Thymol sulfon phthalein, Lubs and Clark (1915).

Thymol nitro sulfon phthalein,[11] Lubs and Clark.

Di bromo thymol sulfon phthalein, Lubs and Clark (1915).

α-napthol sulfon phthalein,[12] Lubs and Clark (1915).

Carvacrol sulfon phthalein,[11] Lubs and Clark.

Orcinol sulfon phthalein, Gilpin (1894).

Of these sulfon phthalein indicators we have chosen the following:—the phenol and tetra bromo phenol, the thymol and di bromo thymol and the o-cresol and di bromo o-cresol sulfon

[11] These indicators were prepared since the publication of our preliminary report on new indicators.

[12] Since the publication of our preliminary report we have received several inquiries concerning α-naphthol sulfon phthalein from those who desire an indicator useful in the alkaline region beyond the range covered by phenol sulfon phthalein. Although the α-naphthol compound may be useful, its solutions decompose so readily that we have entirely abandoned its use. The range within which it is useful is much more satisfactorily covered by other sulfon phthalein indicators.

phthaleins. These form a series useful between $P_H = 1.0$ and $P_H = 10.0$. With the exception of a portion of the range covered by methyl red this series is without serious gaps. A list of the useful ranges is given in table 2.

TABLE 2

List of indicators

CHEMICAL NAME	COMMON NAME	CONCENTRATION	COLOR CHANGE	RANGE P_H
		per cent		
Thymol sulfon phthalein (acid range)....................	Thymol blue	0.04	Red-yellow	1.2–2.8
Tetra bromo phenol sulfon phthalein..................	Brom phenol blue	0.04	Yellow-blue	3.0–4.6
Ortho carboxy benzene azo di methyl aniline.............	Methyl red	0.02	Red-yellow	4.4–6.0
Ortho carboxy benzene azo di propyl aniline..............	Propyl red	0.02	Red-yellow	4.8–6.4
Di bromo ortho cresol sulfon phthalein..................	Brom cresol purple	0.04	Yellow-purple	5.2–6.8
Di bromo thymol sulfon phthalein..................	Brom thymol blue	0.04	Yellow-blue	6.0–7.6
Phenol sulfon phthalein......	Phenol red	0.02	Yellow-red	6.8–8.4
Ortho cresol sulfon phthalein.	Cresol red	0.02	Yellow-red	7.2–8.8
Thymol sulfon phthalein (see above)....................	Thymol blue	0.04	Yellow-blue	8.0–9.6
Ortho cresol phthalein........	Cresol phthalein	0.02	Colorless-red	8.2–9.8

With the improved method for the preparation of the sulfon phthalein indicators described by Lubs and Clark (1915) they may easily be made from materials readily obtained. The indicators can also now be purchased in this country.

SECTION VII. NOMENCLATURE OF INDICATORS

It is desirable, especially in purchasing indicators, that their chemical names should be used, since these names define the composition and to some extent the structure. Nevertheless

some of the chemical names are so verbose that they must inevitably be abbreviated in laboratory parlance. It is therefore desirable to agree upon some abbreviations.

Ortho-carboxy-benzene-azo-di-methyl-aniline is already known as methyl red. For the corresponding di-propyl compound we suggest "propyl red." Following a similar system we suggest for the sulfon-phthalein series of indicators the names which are listed in table 2.

(To be continued.)

SOIL FLORA STUDIES[1]

PART I

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GENERAL INTRODUCTION

During the past twenty years or more, considerable attention has been given to the relation of bacteria to nitrogen-transformations in soil and in manure. This particular phase of soil microbiology has been so extensively studied because of the manifest importance of nitrogen in the nutrition of plants. There still remain, however, many other problems connected with the microörganisms in the soil that are of fundamental importance and which must be solved before a true appreciation of the significance of soil bacteria to agriculture is possible.

There are, for example, many unanswered questions relating to the distribution, function, and associative action of the microorganisms known to be abundant in soil. These questions require a method of attack almost the opposite from that used in studying the nitrogen-transformations. The latter problem started with a known function and was directed toward the unknown organisms to which this function belonged; the other problem starts with known organisms and is directed toward their unknown functions. The study of nitrogen-transformations has been centered around a few special physiological activities; while the other problem is concerned with the general characteristics of the entire soil flora. Therefore, investigations of the latter sort may be distinguished from those of the former by calling them soil flora studies.

In making a study of the soil flora, the first step necessary is

[1] Original abstracts of Technical Bulletins of the New York Agricultural Experiment Station.

classification. Investigations relating to the distribution or function of the microörganisms cannot proceed far without a thorough knowledge of the kinds of organisms in question. It is not yet possible to make a complete classification of the microscopic flora of soil; but it is possible to make a preliminary grouping of the organisms. Nothing more than this is attempted in the present work; but it is hoped that this preliminary grouping may be used by later investigators and developed by them into a more extensive classification.

The results that are now ready for publication are to be embodied in a series of five papers, entitled:

I. The general characteristics of the microscopic flora of soil.
II. Methods best adapted to the study of the soil flora.
III. Spore-forming bacteria in the soil.
IV. Non-spore-forming bacteria in the soil.
V. Actinomycetes in the soil.

The papers are to be published in full as technical bulletins of the New York Agricultural Experiment Station, which are to appear simultaneously with these abstracts in the Journal of Bacteriology.

I. GENERAL CHARACTERISTICS OF THE SOIL FLORA

Previous investigations

Soil flora studies in the past—and the same thing is true concerning flora studies of milk or water—have developed in two different directions, the first extensive, the second intensive. Extensive studies have resulted in learning a little about many kinds of soil organisms; intensive studies in learning much about a few small selected groups of organisms. Both lines of work must be harmonized in order to obtain a comprehensive knowledge of the soil flora.

The most important contributions to the extensive flora studies of soil are those of Chester (1900, 1903, 1904) and of Hiltner and Störmer (1903). The contribution of Hiltner and Störmer is especially important because they pointed out three

large groups of soil microörganisms which may be readily distinguished on gelatin plates and showed that the relative abundance of these three groups is nearly constant in normal soil. From their data it appeared that any external influence which disturbed the equilibrium of the soil flora would be indicated by a change in the relative abundance of these three groups. This conception of the soil microörganisms as being normally in a state of equilibrium has proved of considerable value in interpreting soil phenomena.

Meanwhile intensive work on special groups of soil bacteria has been accumulating more rapidly than extensive work on the general flora. Part of this work has been done on the group of spore-forming bacteria, and part of it on the Actinomycetes. As these two groups are to be taken up in two of the following papers, a more complete discussion of the literature will be given in them.

Thanks to the intensive work which has been done on these two groups, extensive work on the general soil flora can be undertaken today with a much greater chance of success than in earlier years. Flora studies have proved of value in dairy work, having resulted in showing the significance of the lactic acid types in milk and cheese (in milk the common lactic acid type, and in cheese the common type and also the Bulgarian type). Knowledge of equal importance in regard to soil may easily result from a better acquaintance with the soil flora.

Realizing this, the writer several years ago criticized the prevailing methods of soil bacteriological investigations, pointing out the importance of making a flora study, and concluded with the words: "in the future, if we wish to make any great advance in our knowledge, we must learn first to distinguish as rapidly as possible the different bacteria present in any given soil and then must study the significance of the forms we have learned to recognize" (Conn, 1909). Following up the line of work indicated in this statement, a preliminary grouping of the bacteria in a certain soil was published a few years ago (Conn, 1913). This study included all the forms obtained from gelatin plates incubated in the presence of air. About thirty-five types

of bacteria were mentioned that could be distinguished by means of certain cultural and physiological characteristics. It was expected that some of these types could later be broken up into two or more species, while others would prove to be separated by characteristics not sufficiently constant to furnish actual distinctions. This study has been continued for five years since the work already published was brought to an end; and now considerable information is at hand to show which of these types are distinct, which must be grouped together, and which require further subdivision. During the course of this study, several minor points have been investigated which have been published from time to time. The surprisingly high plate counts of frozen soil, for example, were noticed early in the work, and as data accumulated on this subject, they were published (Conn, 1910, 1912, 1914 a; 1914 b). Gradually, as the work progressed, evidence was obtained as to the comparative value of different culture media for soil bacteriological work (Conn, 1914 c). These minor publications all had to do with quantitative work alone, although the main line of investigation was primarily qualitative. The reason for this was the greater ease with which results could be obtained in quantitative work. It is only recently that the qualitative side of the work has been advanced sufficiently for publication. Two short articles have been published within the last year, one dealing with the spore-formers of soil (Conn, 1916 a; 1916 b), and the other with Actinomycetes (Conn, 1916 c; 1916 d). The work published in all these papers formed an integral part of the general soil flora study; so it is all summarized in the present series of publications.

Soils investigated

During the course of this investigation, samples have been taken from as great a variety of soils as could be obtained in the neighborhood of the Experiment Station. This included muck, clay loams, loams, sandy loams, and fine sand. They were mostly agricultural soils, but some of the muck samples were from woodland. The samples were taken from soils in various

stages of cultivation. Many of the samples were so selected as to furnish a comparison between sod and cultivated portions of the same soil.

Kinds of microörganisms in soil

Limitations of the study. At present it would be a hopeless task to make a complete study, or even a complete classification, of the various types of microörganisms occurring in soil. Because of the necessity of setting some limit to the work, only those forms have been studied that grow in the presence of air and occur in appreciable numbers on plates inoculated with soil infusion diluted 100,000 times. This excludes a number of the soil bacteria that have been described by others.

The organisms concerned with the transformations of nitrogen are not mentioned in this work because special media are necessary in order to obtain them. As all the plates were incubated in the presence of air, strict anaerobes, if such occur in soil, have been overlooked; but evidence has been obtained which indicates that they are not of much importance in soil. Two other classes of organisms that may be important in soil, which have been excluded from the present work, are higher fungi and protozoa; but it is still a matter of dispute whether these two groups are sufficiently active in soil to be of importance in comparison with bacteria.

A further limitation of the work has resulted from the fact that a complete classification of a flora as complex as that of soil—or even that part of it which develops on aerobic gelatin plates—offers difficulties that are almost insurmountable at the present day. The most that can be looked for at present is a preliminary classification. This preliminary classification should include descriptions of known species that can be recognized without question; but to describe new species seems unwise. The literature relating to soil bacteriology is full of names of species in regard to which too little information is given to make recognition possible. It seems unwise to increase this confusion by adding more new names. The best plan seems to

be to classify the soil microörganisms into groups or types as small as present methods allow, each of which may be the subject of future investigation. Some of these types are undoubtedly species; others are larger groups, and must be further subdivided in the future.

Peculiarities of the soil flora. One of the most striking characteristics of the soil flora is its uniformity. Quantitatively this uniformity is particularly striking, as the number of colonies developing on gelatin or agar plates, incubated for seven days, is ordinarily between five and fifty million per gram of soil. Qualitatively, there is also considerable uniformity. The characteristic microscopic flora of soil, is different from that of any other natural medium. The following groups of microörganisms almost always develop on aerobic gelatin plates inoculated with soil:

1. From 5 to 10 per cent spore-formers (The *B. subtilis* group). Nearly all the colonies of these bacteria, however, seem to come from spores instead of from active organisms.

2. Under 10 per cent rapidly liquefying, non-spore-forming, short rods with polar flagella (principally *Ps. fluorescens*).

3. From 40 to 75 per cent slowly liquefying or non-liquefying, non-spore-forming, short rods.

4. A few micrococci. In cultural characteristics these are almost identical with the last mentioned group.

5. From 12 to 50 per cent actinomycetes.

Of these five groups, the most important ones seem to be numbers 3 and 5. This conclusion is based upon the following facts: these two groups are always present in large numbers; of the other three groups, the only one always present in large enough numbers to be detected on the plates is number 1, the group of spore-formers, which apparently exists in normal soil only in the form of spores, and hence can not be active there.

The following groups seem to be lacking or to be present in such small numbers that they have been overlooked: organisms producing gas from sugars; non-liquefying bacteria producing acid from sugars; non-spore-formers that can grow in sugar broth in the absence of free oxygen.

II. METHODS BEST ADAPTED TO THE STUDY OF THE SOIL FLORA

Introduction

Methods for use in bacteriological investigations of soil are still in an experimental stage. Various methods have been suggested for different purposes, but none of them have been standardized. For the purpose of making a flora study of soil, it has proved necessary to develop many new methods.

The poured plate of agar or gelatin has been used as the basis of the present flora studies. Plate culture is useful for two purposes: it gives some idea as to the number of microörganisms in the soil; and it serves as a basis for qualitative work.

Plating soil for quantitative purposes

When using the plate method for quantitative work, the important points in technic are the temperature used for incubation and the length of time the plates are incubated. Fairly low temperatures (18°C. for gelatin and not over 25°C. for agar) and at least seven days incubation have been found to give the best results. The exact composition of the medium used (within certain limits) does not seem to have much influence upon the count obtained. This matter was taken up in a previous publication (Conn, 1914 c), in which it was stated that if anything the highest counts were obtained upon a soil-extract gelatin; but an asparaginate agar was especially recommended, principally because of its definite chemical composition. Since that paper was published further modifications of both media have been made.

A number of comparisons have been made between soil-extract gelatin and tap-water gelatin, showing that tap-water can be substituted for soil-extract without affecting the results obtained. Some work has also been done to determine what concentration of gelatin is best. Besides Gold Label Gelatin, which was used throughout the earlier work, two other commercial brands of gelatin have been studied: one prepared for bacteriological work by the United States Glue Company of Milwaukee, the other,

called "Bacto-Gelatin," prepared by the Digestive Ferments Company of Detroit. Both of these brands of gelatin have a higher jellying power than Gold Label Gelatin; so that if they are made up in a strength of 10 per cent about as firm a jelly is formed as with 15 per cent Gold Label Gelatin. The best quantitative results were obtained with 12 per cent United States Glue Company gelatin; but 12 per cent Bacto-Gelatin and 20 per cent Gold Label Gelatin gave high enough counts so that they can also be recommended.

The asparaginate agar has been modified by the addition of 10 grams of glycerin per liter. This was found to favor the Actinomycetes, to stimulate chromogenesis and to increase the size of the colonies to such an extent that it was immediately recognized as an improvement provided it gave a count as high as the unmodified formula. A series of soils was therefore plated on the two media, and the counts were as good or better with the glycerin formula.

As a result of these tests, tap-water gelatin and asparaginate-glycerin agar are recommended for quantitative work. The formula recommended for tap-water gelatin is:

Tap-water, 1000 cc.

Gelatin $\begin{cases} \text{200 grams of Gold Label Gelatin} \\ \text{or 120 grams of Bacto-Gelatin or of United States Glue Company Gelatin.} \end{cases}$

Reaction adjusted to 0.5 per cent normal acid to phenolphthalein. (This will require about 20 to 30 cc. normal NaOH for Gold Label Gelatin, but under 10 cc. with either of the other brands.)

Clarified with white of egg in case Gold Label Gelatin or United States Glue Company Gelatin is used, but unclarified if Bacto-Gelatin is used.

The formula recommended for asparaginate-glycerin agar is:

Distilled water 1000 cc., agar 12 grams, glucose 1 gram, sodium asparaginate 1 gram, glycerin 10 grams, $NH_4H_2PO_4$ 1.5 grams, $CaCl_2$ 0.1 gram., $MgSO_4$ 0.2 gram., KCl 0.1 gram, $FeCl_3$ trace. Reaction adjusted to 1.0 per cent normal acid to phenolphthalein.

Whether the tap-water gelatin or the asparaginate-glycerin agar should be used will depend upon the conditions under which the work is being done. The counts obtained upon the

two media are nearly enough alike so that if a medium for quantitative work alone is desired, the choice between them should rest upon other considerations such as whether low temperatures are available for incubation.

Plating soil for qualitative purposes

When using the plate method as the basis of qualitative work, the composition of the medium is of much greater significance. No single medium has been found upon which all kinds of soil microörganisms can be recognized by means of their colonies. The best procedure at present available for making a qualitative study requires the use of both tap-water gelatin and asparaginate-glycerin agar. In the gelatin medium it is advisable to use 15 to 18 per cent of the Gold Label brand instead of 20 per cent as for quantitative work. On this gelatin the number of colonies of *B. megatherium*, *B. cereus*, *B. mycoides* and *Ps. fluorescens* can be counted. Gelatin also shows the relative numbers of Actinomycetes and lower bacteria, as even the most minute colonies of the Actinomycetes can be distinguished on gelatin (with a low-power microscope) from the colonies of lower bacteria. On asparaginate-glycerin agar it is possible to count the colonies of several types of Actinomycetes, although unfortunately this medium fails to show any difference between two of the most common types. The different kinds of non-spore-formers can not be recognized upon any of the media yet investigated—with the exception of *Ps. fluorescens*, which is not one of the most abundant types.

A long incubation at comparatively low temperatures is so very important in qualitative work that it needs special emphasis. Colonies of the more slowly growing organisms do not begin to appear until the fourth day; counts made on the fourth day or earlier include only a very small proportion of the Actinomycetes and almost none of the large group of slow-growing non-spore-formers. A qualitative study of soil bacteria based on plates incubated for such a short time would give a very erroneous conception of the actual soil flora.

For a complete qualitative analysis of the soil flora, at least three media may prove necessary in plating, one of them adapted to bring out the distinctive characteristics of the common soil types of each of the three groups of soil microörganisms. By plating soil on three such media, it would then be possible to recognize at a glance the most abundant types. The types that can not be recognized by means of their colonies should be isolated and studied in pure culture.

The study of pure cultures

Pure culture studies of about a thousand cultures isolated from soil have been made in the course of the present work. Various methods have been used in these studies. The classification card of the Society of American Bacteriologists has proved quite well adapted to the study of the spore-formers; and has proved of value in making a preliminary study of other organisms before learning what special tests were best adapted to them.

Certain of the tests called for in studying an organism by means of the classification card have appeared to give sufficiently consistent results to be of diagnostic value. These are: shape of vegetative forms; size of vegetative forms; arrangement of flagella when present; presence of spores; size and shape of spores; growth in the absence of oxygen; liquefaction of gelatin; nitrate reduction; chromogenesis; and form of growth in solid or liquid media. Even in regard to these points, however, inconsistent results are often obtained, a fact which must be taken into consideration when using the data as a basis for classification.

Other tests called for on the classification card have proved to give such inconsistent results as to be of little diagnostic value. These are: presence or absence of flagella; fermentation of sugars; diastatic action on potato starch; fermentation of glycerin; production of indol; and action on milk. In some cases it is possible that the inconsistent results of these tests are due to actual physiological variations in the bacteria; but it is felt that more often they are caused by imperfections in the technic at present available for making the tests.

The standard tests have proved of least value for the study of the Actinomycetes and slow-growing non-spore-formers. These two groups grow very poorly in ordinary media and nearly always give negative results in the tests called for on the classification card. In studying the Actinomycetes the most important information was obtained from a study of their growth on various special agar media containing little or no protein material. No methods have yet been found which prove satisfactory for the qualitative study of the slow-growing non-spore-formers.

(To be continued.)

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THE INFLUENCE OF MILK AND CARBOHYDRATE FEEDING ON THE CHARACTER OF THE INTESTINAL FLORA

IV. DIET VERSUS BACTERIAL IMPLANTATION

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This paper marks the culmination of several years of investigation carried on in this laboratory on the relation of diet to the intestinal flora. The results given in earlier publications (Hull and Rettger (1914); Rettger and Horton (1914); Rettger, (1915) may be summarized briefly as follows.

1. A change in the diet of white rats from the ordinary mixed food to a special diet containing starch, lard, protein-free milk and a pure protein quickly resulted in a marked simplification of the intestinal flora, the Gram-positive organisms increasing, often to the extent of constituting the entire flora. *B. coli* and other intestinal organisms were practically eliminated, while bacilli of the type of *B. acidophilus* (Moro) predominated or were present in such numbers as to exclude all other forms.

2. *B. acidophilus* was found to be a common inhabitant of the intestinal tract of white rats under certain conditions of diet. It was increased in numbers by grain feed (particularly oats, wheat and corn), milk, and lactose. Its preponderance over other organisms was brought about within a period of two to four days when lactose was fed. It was often supplanted by *B. bifidus* which on continued lactose feeding persisted. In milk feeding the acidophilus phase was more permanent, and *B. bifidus* seldom gained the ascendency. Carbohydrates, other than milk sugar, failed to bring about this transformation.

The ingestion of foreign bacteria, even in large numbers, did not of itself bring about an elimination or displacement of the common intestinal microorganisms. This was especially true of *B. bulgaricus*. Vastly more important is the influence of diet, particularly milk and lactose. The feeding of *B. bulgaricus* tablets without the use of milk or lactose can therefore be of little, if indeed of any, value.

The work upon which this paper is based was largely a continuation of the earlier experiments on milk and carbohydrate feeding in which white rats were used almost exclusively. Particular emphasis was placed, as heretofore, on organisms of the aciduric group of bacteria, especially *B. acidophilus* and *B. bifidus*. Numerous comparative tests were made with these two organisms and *B. bulgaricus*. Feeding experiments were conducted on man as well as rats. More extended studies were made of the influence of a starch diet on the intestinal bacteria, and on the amylolytic or starch decomposing bacteria, particularly *Glycobacter peptolyticus* of Metchnikoff. Furthermore, tests were made for the presence of lactose and increased acidity in the digestive tract, after carbohydrate feeding.

In the present investigation 180 individual experiments were conducted on white rats, four on man, and six on other laboratory animals. In many instances the same animals were used more than once.

A more satisfactory method of collecting the feces of white rats was employed than has been described in previous papers. The rats were held by the tail and gently rubbed on the back just above the base of the tail. In this way the samples could be collected into test tubes containing sterile water or physiological salt solution. The methods of feeding, and the technique in general were essentially the same as those used heretofore.

For the detection of starch-attacking organisms, starch-agar plates were used, as originally suggested by Eijkmann (1901). This medium at first contained 0.1 per cent each of peptone and meat extract, but later both were omitted, the only ingredients being 1 per cent each of corn starch and agar. The starch

digesters on the plates showed a clear zone around the colony which failed to turn blue on the application of iodine solution (Lugol). Tests for the Welch bacillus were made by inoculating whole milk and heating the tubes at 80°C. for fifteen minutes. Sufficient anaerobiosis was provided for by the cream layer. *B. coli* was detected by its colonies on the agar plates and by gas formation in Veillon tubes. Lactose bile tubes were also used at times, as well as the litmus-lactose-asparagin-agar medium recommended by Ayers (1915).

The diet of the rats, unless otherwise noted, consisted of bread and green or vegetable food. Milk was supplied in small dishes, and the lactose and other carbohydrates were usually given in dry form on the bread or bread crumbs. The bacteria which were fed were either suspensions of twenty-four slant glucose agar growths in water or, as in the study of the glycobacteria, plain bouillon cultures or the washings of plain slant agar growths.

MILK FEEDING

In the later experiments conducted on white rats results were obtained which were in full agreement with those already published. In addition to the ground covered heretofore the influence of milk feeding on the intestinal flora of rats which were supplied with a high meat diet was determined. The results of these experiments may be stated as follows:

1. Sweet milk when added to a diet of bread and green food or vegetables favors the multiplication of the aciduric group of bacteria in the intestine of the white rat. During long continued milk feeding *B. bifidus* of Tissier may be present in large numbers, but as a rule *B. acidophilus* of Moro is the predominating organism.

2. While a marked transformation of the flora takes place complete simplification is rarely brought about by the use of milk alone.

3. The addition of milk to an established high meat diet does not lead to the multiplication of the aciduric group of bacteria to such an extent that the so-called "putrefactive" bacteria (*B. coli* and *B. welchii*) are eliminated.

CARBOHYDRATE FEEDING

Some of these experiments were merely a repetition of the earlier work on carbohydrate feeding, and were entirely corroborative. In all of the work on carbohydrate ingestion no other sugar besides lactose was found to exert any apparent influence on the intestinal bacteria, with the exception of dextrine, and in only two instances was any change brought about by dextrine. In these rats, C20 and C21, the flora was by no means simplified, nor did *B. acidophilus* appear prominent. In the smears there were seen, however, short thick rods with pointed ends which were Gram-negative, and which were in marked contrast to the flora at all other times. The remaining six rats which received dextrine did not show this change.

In all of the work on lactose feeding only one rat failed to respond in the usual way. During a period of thirty days while the lactose was supplied neither *B. acidophilus* nor *B. bifidus* could be found in the smears, plates or Veillon tubes made from this animal. The results with all of the other rats which were fed lactose were quite consistent, as in previous investigations. *B. acidophilus* soon made its appearance, and unless supplanted by *B. bifidus*, during prolonged lactose ingestion, it remained the predominant, and as a rule practically the only organism occurring in the feces. In a number of instances, however, *B. bifidus* assumed the chief rôle of importance.

LACTOSE VERSUS A HIGH MEAT DIET

White rats which had been on a meat diet (10 to 15 grams of chopped beef daily) were given the customary amounts of lactose, 4 to 5 grams per day. These rats at the beginning of the lactose feeding harbored a typical meat diet flora, *B. coli* and *B. welchii* being prominent, and *B. acidophilus* apparently absent.

In rat B7 which had been on the meat diet for nine days before the lactose was fed a gradual simplification of the intestinal flora took place, and by the twelfth day practically no organisms were present but *B. acidophilus*. The meat and lactose feeding were continued for one month, at the end of which time

the rat was killed. *B. acidophilus and B. bifidus* were found to be present throughout the length of the intestine, but, in the colon a few other forms were observed.

Rat B9 having been on the meat diet for two months was given lactose for eight days. There was a change from the typical mixed meat flora to one in which *B. acidophilus* predominated, although a few other forms, particularly *B. coli*, were still present. On post-mortem examination *B. acidophilus* was found almost pure in the upper small intestine, but mixed with other forms of bacteria in the ileum and colon.

Rat B19 was fed nothing but meat for ten days, after which it received a meat-lactose diet for eighteen days. Within five days *B. welchii* was eliminated and the other organisms reduced in numbers, even *B. çoli* being detected with difficulty. The feces contained *B. acidophilus* in almost pure culture. Rat C23 having received nothing but meat for nine days, was placed on a meat-lactose diet for thirty-five days. The fecal flora changed from a strictly meat to the lactose type, but at no time were gas formers absent from the Veillon tube.

Rats C4, C7 and C10 were given meat for eleven days, the intestinal flora changing to the high meat diet type. On the addition of lactose to the food there was a marked change toward the aciduric type of bacteria, although *B. coli* was still present at the end of a week. Rats C16 and C17 were kept on a meat diet for two weeks, at the end of which time a typical meat flora was established. On changing the diet to one of lactose and bread a change to the lactose type of fecal bacteria occurred.

In a number of instances lactose was first supplied to the rats, and as soon as an aciduric flora was established meat was added to the diet in the amounts stated before, in place of the bread. Rats C5 and C6 having gone on a lactose-bread diet for twelve days, received meat in place of the bread. During the two weeks that the meat was fed little change took place in the fecal flora, although a small number of colon bacilli were detected from time to time. On discontinuing the lactose feeding there was a rapid change in the flora to the mixed type. In

rat C8 which had been fed a lactose-bread diet for five days a non-aciduric flora was established after there was a change to a strictly meat diet.

MINIMUM LACTOSE REQUIREMENTS

The amounts of lactose required to bring about complete transformation of the flora varied with the different animals. As a rule the amount of lactose contained in the milk which was consumed was not sufficient. Few of the animals took more than 20 cc. a day, and some took less than half of that quantity. The total amount of milk sugar consumed was from about 0.5 to 1 gram daily.

Five rats which had been on a high lactose diet for some time were given 1 gram of the sugar daily with 12 grams of bread crumbs. After twelve days one rat had a mixed flora, although acidophilus bacilli were still numerous. In the remaining five animals there was a less pronounced change, *B. acidophilus* and *B. bifidus* still being the predominant types. After increasing the daily amount of lactose 1 to 2 grams per week there was an almost complete reversion to the pure, simplified type.

Two rats which had been on the regular stock diet were each given 2 grams of lactose with 10 to 12 grams of bread crumbs daily. The typical mixed diet flora was transformed in a few days to one of an almost purely aciduric character. It appears, therefore, as if 1 to 2 grams of lactose per day are sufficient to bring about simplification, although complete transformation requires amounts somewhat in excess of 2 grams.

THE FATE OF LACTOSE IN THE INTESTINE OF THE WHITE RAT

A number of rats which had been on a high lactose diet (dry) for at least three or four days were killed and thorough examinations of the intestine made for the presence of acids and unchanged lactose. For this purpose samples of intestinal contents from different portions of the intestine were weighed, emulsified in distilled water and tested as to acidity and milk sugar. For determining the acidity the suspensions were ti-

trated against phenolphthalein with twentieth normal sodium hydroxide. No unusual acidity of the intestine could be noted (see page 55).

In the first tests for sugar the emulsions were filtered and the Fehling test applied to the filtrate. The results were negative. In the next experiments the samples of well shaken material from the intestine were boiled vigorously before filtering, and relatively large amounts of the filtrate employed for the Fehling test. The results were, with very few exceptions, positive. According to these results the lactose must have been present in the intestine in a solid or undissolved form, and required heating to bring it into solution so that it would appear in the filtrate in sufficient amount to give an unmistakable reaction with the Fehling solution.

Sugar was found in different parts of the intestine of rats to which it was fed in dry form; also at times in the voided feces. Hence, the conclusion may be drawn that in the feeding of a high lactose diet some of the lactose is not absorbed from the intestine, and that the unabsorbed remnant serves as favorable food for the aciduric type of bacteria. This view seems justified, at least in so far as the feeding of lactose in the dry form is concerned.

Several experiments were conducted on rats which received the lactose in concentrated aqueous solution (one part of lactose to seven parts of water) in daily amounts containing 4 to 5 grams of the sugar. When a typical simplified flora was established and within three to four hours after lactose feeding the animals were killed. Without exception, lactose was found in the intestine. Unboiled filtrates from the stomach and jejunum contents of one rat gave positive Fehling tests, while those from the duodenum, ileum and colon were negative. Boiling of the suspension from the last three sources made no difference in the findings. Similar results were obtained in a second rat, with the exception that the lactose was found as far down as the ileocecal valve. In two other rats sugar was detected in solution in the stomach, jejunum and ileum, but not in the duodenum and colon.

According to these results lactose, when fed in solution, is absorbed from the digestive tract before it reaches the lower intestine. Therefore, if lactose is acted upon by the aciduric group of bacteria, the decomposition must take place above the colon, unless such small amounts of lactose reach the colon as to escape detection by the method employed.

The following conclusions may be drawn regarding lactose feeding:

1. The ingestion of lactose in sufficient amounts will completely simplify the intestinal flora of the white rat, reducing it to the purely aciduric type.
2. The simple character of the flora remains permanent during continued lactose feeding.
3. The results are the same whether lactose is fed in a dry state or in solution. When dry sugar is employed it may be detected throughout the length of the intestine and in the feces, by means of the Fehling test. When fed in solution, however, its presence could be demonstrated only as far as the ileocecal valve.
4. Whether the regular diet is of the usual mixed type or very rich in protein, as for instance meat, lactose, when fed in large enough amounts, will exert the same simplifying action on the bacteria of the intestine. The putrefactive influence ordinarily brought about by a rich meat diet is, in the white rat, entirely neutralized by the lactose feeding.
5. The daily amount of milk sugar required to bring about a complete simplification of the flora varies somewhat with the individual rats. The average quantity, however, when a mixed diet is supplied is between 2 and 3 grams.

THE INFLUENCE OF LACTOSE FEEDING ON THE CHEMICAL REACTIONS OF DIFFERENT PARTS OF THE DIGESTIVE TRACT

In the experience of the writers all strains of acid-tolerant intestinal organisms are low acid producers, at least as compared with a typical strain of *B. bulgaricus*, and may be classed with the group of lactic acid bacteria designated the "Paratype"

or Type "B" of White and Avery (1910). *B. bifidus* has seldom produced an acidity in excess of 0.7 per cent, while the maximum for *B. acidophilus* has on some occasions reached 1.5 per cent. *B. bulgaricus* Massol, or Type "A," frequently attains a maximum acidity in milk of at least 3 per cent.

Acidity tests were made on the intestinal contents of 29 rats, 17 of which had been on the regular diet and possessed the usual mixed flora, while the remaining 12 had been fed lactose and harbored a simplified flora. Different parts of the digestive tract were tested by the method already described on page 53, in connection with the Fehling reaction for sugar.

While there were differences in the acidity of the individual rats, the acidity curve of the lactose animals runs practically parallel with that of the rats having the usual mixed diet. However, the average acidity of the 12 lactose-rats was less than that of the other group, which is contrary to what might be expected. It is safe to conclude from these experiments, that the acidity in the digestive tracts of the rat is not increased by lactose feeding under the conditions of these experiments.

INFLUENCE OF LACTOSE FEEDING ON MAN

Since the digestive processes of mammals are more or less alike, the ingestion of milk sugar in man might be expected to have the same effect upon the intestinal bacteria as in the rat. This was found, in a measure at least, to be true.

Four human adults consumed milk sugar with the daily diet for varying lengths of time. The diet varied, but in every instance the protein intake was reduced below the average, and usually considerable milk was added.

Subjects H1 and H2 were on the lactose regimen for eleven days. The average meal consisted of two slices of bread with butter, one quart of milk and all the lactose that the subjects cared to consume. Very little fruit was eaten. During the first two days four moderate doses of Epsom salts were taken by H1, but without any resultant diarrhea. During the eleven days this subject consumed 8 pounds of milk sugar. At the begin-

ning of the experiment the intestinal flora was typical of a healthy individual on a mixed diet, no acidophilus bacilli being demonstrable. On the third day the flora began to change, as evidenced by microscopical examination of slides, large Gram-positive rods becoming more numerous. On the seventh day the change was quite marked, acidophilus bacilli making their appearance in the agar plates. By the tenth day *B. acidophilus* was quite plentiful in the plates, with few other organisms accompanying it. *B. bifidus* was present in large numbers in the Veillon tubes, while on the slides large Gram-positive rods made up almost the entire microscopic picture.

Subject H2 consumed only 4 pounds of sugar in the eleven-day period, and took no laxative. On the third day after the beginning of the experiment a few acidophilus-like rods appeared in the smears, but their increase in number was very slow. By the eleventh day, however, the flora became somewhat simplified, with *B. acidophilus* occurring in moderate numbers, but no indication of *B. bifidus* colonies in the Veillon tubes.

The diet of the other two subjects could not be so easily controlled. H3 consumed a small amount of lactose and milk for two weeks, with his regular diet. There was but little change in the flora. For the next two weeks large amounts of lactose and milk were taken, other food being reduced in proportion. A marked change took place in the character of the feces and in the flora. The feces became light yellow in color, with a butyric consistency, and *B. acidophilus* became quite apparent in the smears and on the plates.

H4 on a mixed low protein diet, consumed four pounds of lactose during a period of two weeks. By the end of the first week *B. acidophilus* appeared both in the smears and on the plates. During this week a laxative had been taken causing some diarrhea.

In the following experiment the influence of a laxative upon the intestinal flora is brought out still better. Subject H1 on an ordinary mixed diet with low protein intake, consumed three pounds of lactose a week for a period of one month. Aciduric organisms (acidophilus and bifidus) were easily demonstrated

microscopically and culturally. Following the use of laxatives these organisms were present in much larger numbers. Without the ingestion of lactose no such changes were observed.

It seems probable that the laxative hurried the lactose along so that a sufficient amount of it reached the lower parts of the intestine where the aciduric bacteria multiply most abundantly under the right conditions of nutriment and environment. Without the use of a laxative or some other agent which saves the lactose from rapid absorption in the small intestine but little of the sugar reaches that part of the intestine in which the most marked bacterial changes, putrefactive and otherwise, take place and hence little encouragement is given to the aciduric type of bacteria.

The above results are somewhat analogous to those of Tonney, Caldwell and Griffen (1916) who, in the examination of typhoid carriers found that the typhoid bacillus is more easily recovered from the feces after the use of a cathartic. They appear to be strongly of the opinion that typhoid bacilli are destroyed in the intestine unless hurried along by increased peristalsis.

INFLUENCE OF STARCH FEEDING ON THE INTESTINAL FLORA

In the earlier experiments no perceptible change was observed in the character of the intestinal flora after the ingestion of moderate amounts of starch (1 to 2 grams daily). Since then similar experiments have been conducted in which larger quantities (4 to 6 grams) of starch were fed and in which starch-agar, in place of the regular agar or glucose-agar, plates were employed for the detection of amylolytic bacteria.

Rats C41 and C42, having previously been on the stock diet of bread and vegetables, were supplied with liberal amounts of corn starch together with ground bread. At the beginning no starch-attacking colonies were obtained from the feces. Within four days the plates showed numerous colonies of amylolytic organisms with their characteristic clear zones which on iodine treatment remained colorless. Rat C17 was given a mixture of corn starch and ground bread, equal parts by weight, for fif-

teen days. No starch decomposing bacteria could be demonstrated in the feces at the beginning. Within three days, however, numerous colonies of this type made their appearance on the plates. These organisms remained conspicuous throughout the experiment. In three to four hours after the last starch feeding the rat was killed. Starch was found in the stomach, but not beyond. On microscopical examination of material from the intestine the ordinary mixed flora of stock rats was observed.

Rats C5 and C6 were supplied with corn starch and bread for 18 days, and gave practically the same results. Rat C37 received the same diet for 25 days. Amylolytic bacteria soon became apparent and remained prominent throughout the period, as shown by the starch-agar plates.

Control rats which were on the regular stock diet at no time harbored this type of bacteria in sufficient numbers to be detected by the plate or any other method. Nor was any such tendency shown during corn and wheat feeding. However, one of two rats given wheat flour yielded a considerable number of typical starch attacking colonies.

The above positive results were obtained with the starch-agar plates only. By the use of ordinary agar or glucose-agar plates little difference could be seen between the flora of stock and of starch-fed animals. Direct microscopic examinations also revealed little if any difference. The conclusion must be drawn, therefore, that the ingestion of starch, while it brings about a marked increase in the strongly amylolytic type of intestinal bacteria, does not materially alter the relative numbers and distribution of the common intestinal organisms. However, the results are such as to show that the feeding of starch has a more marked influence upon the flora than the mere feeding of large numbers of representative starch decomposing bacteria, of which *Glycobacter peptolyticus*, which has been employed by us, is a notable example.

GRAIN FEEDING

In these experiments 35 white rats were employed; wheat was given to 5, oats to 8, corn to 9, mixed grain feed to 5, and wheat flour to 5. The individual experiments lasted from a few days to three and a half months. A small number of tests were also conducted on the common domestic fowl. Without going into the details of this particular investigation the conclusions may be stated as follows:

The ingestion of wheat and of oats seems to favor in a small degree the multiplication of *B. acidophilus* in the intestine of the white rat. In no instance was *B. acidophilus* predominant, nor did the flora of the rats become appreciably less complex.

B. acidophilus is more or less abundant in the intestine of the common domestic fowl, and the temporary substitution of one grain in the mixed feed for another has but little influence upon the intestinal flora. Addition of milk, however, tends materially to increase the number of the aciduric bacteria.

Corn exerts little if any influence on the character of the intestinal flora. Wheat flour is likewise of little significance.

Mixed grains (wheat and oats, or wheat, oats and corn) affect the intestinal organisms in about the same manner and degree as oats or wheat, when these are fed alone.

INGESTION OF FOREIGN BACTERIA AND INTESTINAL IMPLANTATION

Repeated attempts to establish *B. bulgaricus* in the intestine by the ingestion of large numbers of these organisms were unsuccessful. Some of the decisive results obtained in earlier experiments have already been published, and little need be added here except that these results have been consistent throughout the investigation, and admit of but one conclusion, namely that the mere feeding of *B. bulgaricus* does not lead to the acclimatization of the organism in the intestine, even for a short time. The ingestion of milk, raw or sterile, brought about a change in the aciduric type of flora, but this type was represented by *B. acidophilus* of Moro, and not by the bacillus of Massol, as was readily shown by certain cultural tests, par-

ticularly the character of the agar colonies, and by the slowness with which litmus milk was acidified.

Similar experiments were conducted with *Glycobacter peptolyticus*, an active starch-digesting organism which was originally isolated by Metchnikoff from the intestine of a dog, and which has been advocated by Metchnikoff and others as an important aid in bringing about the acclimatization of *B. bulgaricus* in the intestine. It is claimed that this organism attacks the starches and thus makes them available for utilization by *B. bulgaricus* (Wolman, 1912).

Several experiments involving the use of *Glycobacter peptolyticus* were carried on with white rats. As this organism readily produces spores difficulty was at first encountered in the interpretation of results. It was but natural to expect spores to be recovered from the intestine, even though very young cultures were fed.

SPOROGENOUS CULTURES OF GLYCOBACTER PEPTOLYTICUS

As a rule abundant spore formation took place in cultures of *Glycobacter peptolyticus* within twenty-four to thirty-six hours when the organism was grown at 37°C. on plain agar. Water suspensions of the bacilli and spores were prepared from forty-eight hour slant agar tubes, and poured over the bread which constituted the chief diet of the rats. The feces were examined at regular intervals. In order to distinguish between vegetative and spore forms of the glycobacter two sets of tests were made; in one of these the fecal suspensions were heated at 85°C. for fifteen minutes, while in the other the heating was omitted.

In the first experiment two white rats were employed. During the twelve days that the experiment continued there was a small number of amylolytic organisms on the starch-agar plates. The colonies from the unheated suspensions were but slightly in excess of those obtained after preliminary heating.

In the second experiment four rats were fed glycobacter suspensions for five days. A small number of starch-decomposing organisms was obtained from the feces. Heated and unheated

suspensions showed some differences, however. In most instances the counts on the plates of the latter were much lower than on the other set, the reduction amounting to as much as 17 to 1, 24 to 3, and 20 to 2. Vegetative forms were undoubtedly present in the feces.

In the third set of experiments eight rats received spore suspensions for eleven days. At the end of this period glycobacter was present in the feces in comparatively large numbers, and mostly in the vegetative form. It did not persist long, however, as in three days after the spore feeding was discontinued very few colonies of starch attacking bacteria could be obtained from three of the rats, while in the feces of the fourth no organisms of this type were demonstrable.

Since very few or no bacteria which are able to decompose starch were present in the feces of the above animals before the beginning of spore feeding, it must be concluded that development of at least some of the spores must have taken place in the intestine, presumably the large intestine. Whether any multiplication of the organism occurred in the intestine is wholly a matter of conjecture. It may be assumed, however, that the organism is able in the spore form to pass through the stomach and small intestine without serious injury, especially when the diet consists essentially of bread, which contains considerable starch. The above experiments are incomplete, and are to be resumed.

FEEDING OF ASPOROGENOUS SUSPENSIONS OF GLYCOBACTER PEPTOLYTICUS

Non-sporing growths of this organism were easily obtained by cultivating it on glucose agar. Suspensions of twenty-four hour asporogenous cultures were fed with the regular diet of bread and vegetables.

Rat B3 received large numbers of the organism poured over bread crumbs. The starch-agar plates showed a marked increase in the number of starch-digesting colonies. Somewhat similar results were obtained with rat C16. On the other hand, rat

B17 which was on a continued high meat diet and which received the same amount of bacterial suspension showed no increase in the number of amylolytic organisms in the course of five days of continued feeding. The small number of colonies of starch decomposing bacteria which appeared on the plates before the ingestion of the bacterial suspensions remained constant or was slightly reduced during the investigation.

Rats C1 and C2 were fed Glycobacter with bread crumbs for six days. No amylolytic organisms could be demonstrated in the feces before the ingestion of the glycobacterium. Following this, however, starch-attacking bacteria appeared in varying numbers, though they were never numerous.

In the next experiment four rats were given suspensions of Glycobacter for five days. During this period two of the rats yielded a small number of colonies on the starch-agar plates, but for only one day. In the feces of the other two rats no organisms of this type were observed.

Finally, twelve rats were employed in a series of tests. They were given asporogenous cultures, as before, daily for twelve days. During this entire period *Glycobacter peptolyticus* failed to establish itself in the intestine, as shown by the starch-agar plates, although on the eleventh and twelfth days a few starch-decomposing colonies were obtained. Two days later, however, no colonies appeared.

While the above results are not consistent they indicate that the mere feeding of *Glycobacter peptolyticus*, even in enormous numbers, does not bring about an established flora of starch-decomposing bacteria in the intestine of the white rat. In the later experiments the possible occurrence of spores was given greater consideration than before. All feedings were controlled by the previous microscopic examinations of the suspensions as to the presence of spores. Furthermore, it should be borne in mind that the diet in all but one of these experiments consisted almost entirely of bread which is rich in starch, and that on this diet, without the addition of bacterial cultures, few if any starch-attacking bacteria were present in the feces. The establishment of bacteria of this type should be made relatively

easier when large numbers of the organisms are ingested. In the single experiment in which meat was almost the entire diet no colonies whatever of amylolytic-bacteria were obtained from the feces either before or during the period of bacteria administration. These experiments are to be resumed, in order to obtain more conclusive results as to the fate of starch-decomposing bacteria under different conditions of diet, when introduced into the digestive tract.

Very definite results have been obtained with *B. coli*. This organism, although a normal inhabitant of the intestine of man and animals, does not increase appreciably in numbers in the intestine when ingested in large quantities. Rats A15 and A26, the intestinal flora of which had been simplified by continuous lactose feeding, were given, in addition to the lactose, twenty-four hour slant agar cultures of *B. coli* daily for a period of two and three weeks respectively. At no time were *B. coli* colonies obtained on the agar plates, nor was the simplified character of the flora of the intestine changed, in so far as microscopic examination could demonstrate. Even in a rat which had not received lactose for three weeks and which possessed the usual mixed flora, liberal feeding of *B. coli* for four days did not bring about a perceptible increase in the numbers of this organism. These findings were further corroborated by bacteriological examinations of the stomach and intestine of each of these animals, and are in full accord with those of Rettger and Horton (1914) and of Seiffert (1911).

Another well-known organism which is often found in the intestine, *Proteus vulgaris*, was employed by us in the same way. Two rats (C4 and C7) which had received no other diet besides bread and vegetable food, and the flora of which was of a typical mixed character, were fed large numbers of the organism in water suspension administered with the food, or by the pipette method. Very few of the organisms fed could be recovered from the feces by the usual method now adopted for this organism (gelatin tube culture and subsequent inoculation of slant agar condensation fluid). Even the substitution of meat for the bread in the diet did not make any material difference.

In his experiments on man and rabbits Kulka (1914) demonstrated that *B. metchnikovi* and *B. prodigiosus*, when introduced, either by mouth, or subcutaneous, intravenous or intraperitoneal injection, do not appear in the feces. Mitchell and Bloomer (1914) obtained similar results in the common domestic fowl with the typhoid bacillus.

Raubitscheck (1912) obtained positive results by first immunizing animals with the organism in question, by subcutaneous or intraperitoneal injection. When, after such preliminary treatment, the animals were given the organisms *per os* the bacteria were recovered in large numbers from the feces. He employed *B. prodigiosus*, *B. kiliense* and *M. cholerae*.

Similar experiments were carried on by the writers with white rats and guinea pigs. *B. acidophilus* and *B. bulgaricus* were used as the test bacteria. These were grown on slanted glucose agar and washed off with sterile water. Rat B3 was given four subcutaneous injections of *B. bulgaricus* suspensions in 1 cc. quantities at intervals of seven days. Rat B17 received four subcutaneous and three intraperitoneal injections of *B. acidophilus* in amounts varying from 0.5 to 1.5 cc. Rat B19 was given the same number of injections of another strain of *B. acidophilus* in amounts varying from 0.5 to 1.5 cc. Rat B19 was given the same number of injections of another strain of *B. acidophilus*, while a single guinea pig received the same treatment in which suspensions of *B. bulgaricus* were employed. Checks or controls were kept in the cages with each of these animals.

Within a few days after the last injection the different animals were fed suspensions of the same organisms with which they had been immunized. The results were uniformly negative, in so far as any appreciable change in the intestinal flora was concerned. However, since macroscopic and microscopic agglutination tests were negative, too much significance must not be attached to the results of this series of experiments. The organisms employed may not have been adapted to this line of investigation; or, the immunization of the animals may have been far from complete.

GENERAL DISCUSSION AND CONCLUSIONS

B. acidophilus (Moro) and *B. bifidus* (Tissier), two of the well-known members of the aciduric or lactic acid bacillus group of bacteria, are common inhabitants of the intestinal tract of the white rat and of man. At times their numbers may be so small that they escape detection without a most thorough search. Again, they may be very abundant and establish themselves for at least short periods of time to the exclusion of all other forms. The typical flora of infants which subsist on mothers' milk is a good illustration of the extent to which one intestinal organism (*B. bifidus*) may dominate and even supplant all other types. The most important factor in determining the character of the flora is the diet.

Lactose, milk and mixed grains (wheat, oats, etc.) are specific articles of diet which exert an influence on the intestinal bacteria. Lactose, when fed in sufficient quantities (two to three grams daily), brings about a complete transformation of the flora of white rats within two to three days: milk requires a longer time, and does not bring about a complete change. Milk and lactose together form the most practical and effective diet, at least for man. Grain feeds tend to increase the number of aciduric bacteria, but their influence is comparatively small.

Milk undoubtedly owes its beneficial action to the lactose which constitutes almost half of the solid matter present. The explanation of this action must lie in the fact that the lactose is absorbed slowly from the intestine. On several occasions it has been found in the feces of rats that had been supplied with it as a part of their diet. The raw grains are also probably acted upon slowly, or at least some of the intermediate carbohydrate products are not immediately absorbed. Bread, on the other hand, which contains cooked starch does not foster the development of the aciduric bacteria, because it is digested quickly, and no available sugar remains in the intestine long enough to be attacked and utilized by this group of bacteria.

The most satisfactory explanation of the favorable influence of lactose on the aciduric bacteria of the intestine must be

found in the fact that when lactose is present, even in minute quantities, optimum cultural and environmental conditions are created for these particular organisms, without a corresponding favorable change for the mass of other bacteria ordinarily present. That the action could not have been due to lactic acid produced from the sugar is strongly indicated by the absence of increased acidity in the intestine of the lactose rats, as compared with the controls. Rovighi (1892) showed that lactic acid, when ingested, had only a slight influence on intestinal putrefaction. Winternitz (1892) claimed that lactose when fed to animals or man exerted an inhibitory influence on putrefaction, but that this action was not due to lactic acid formed from the sugar, but to the lactose itself.

Meat or other high protein diet increases the indol-producing bacteria and other organisms of the so-called "putrefactive" type, like *B. coli* and *B. welchii;* cornstarch appears to foster the amylolytic group of intestinal organisms, while in a few experiments grain feed seemed to favor the development of what appeared to be fusiform bacilli.

The reaction of the intestine remained independent of the character of the intestinal flora. While the acidity of the intestinal contents varied in different rats, it was not increased during the course of lactose feeding experiments. The acidity was highest in the duodenum, as a rule, and lowest at the ileo-cecal valve.

It has been conclusively demonstrated in our work that a high lactose diet markedly influences the intestinal flora of man. These results have been fully confirmed recently by other investigators. Barker (1914) and Torrey (1915) report favorably on the use of lactose and milk in typhoid fever. Torrey demonstrated that the feeding of a high carbohydrate diet (milk and lactose) to typhoid patients tended to reduce the putrefying types of bacteria, and to encourage the acidophilic forms. Plain milk feeding was not as apt to do this as lactose. At least 250 to 300 grams of the milk sugar were required in most instances (daily) to produce a marked change in the intestinal flora of the patients, especially when the initial flora

was of a distinctively putrefactive type. In persons having non-putrefactive organisms predominant at the outset a radical change to an aciduric type was readily brought about by lactose ingestion.

The acclimatization of *B. bulgaricus* in the intestine after oral administration is a much-disputed point. The claims of Metchnikoff and his followers (Cohendy, 1906; Belonowsky, 1907, and Leva, 1908) that the ingestion of *B. bulgaricus* brings about an implantation of the organism in the intestine have not been substantiated by us. In our earlier work, as well as in the present investigations, we have been unable to establish this organism in the intestine of white rats even for short periods of time, although the bacilli were fed in large numbers.

Luerrsen and Kühn (1908) failed to implant *B. bulgaricus* in the intestine of man by the continued use of yoghurt. Similar results were obtained by Distaso and Schiller (1914) on white rats, although Distaso (1913–14) had observed in persons with cecal or ileum fistula that *B. bulgaricus* was present in the intestine in large numbers twenty-four hours after the use of milk which was soured with *B. bulgaricus*.

Rahe (1915) was unable to obtain evidence by feeding experiments that this organism can become adapted to the lower intestine of man. In monkeys *B. bulgaricus* appeared to be capable of a limited survival in the upper intestine. In a single experiment on a monkey Herter and Kendall (1908) found that by feeding the animal for two weeks on milk fermented with the bacillus of Massol an acid reaction was maintained throughout the intestinal tract, but that the bacillus in question failed to predominate. It was present, however, below the ileo-cecal region and in the duodenum and cecum, in almost pure form.

Attempts to establish *B. bulgaricus* in the intestine after immunization with the same organism were unsuccessful. Thus, claims made by Raubitscheck (1912) for other organisms have not been substantiated by us with the bacteria employed.

Glycobacter peptolyticus fails to establish itself in the intestine of the white rat, even when fed in large numbers. The bacilli may occur in varying amounts in the intestine when the food

consists largely of starch, as for instance bread; but on a rich protein diet very few are recovered from the feces. Spores of *Glycobacter peoptolyticus* may pass through the stomach and the small intestine unharmed, and may even undergo development into the vegetative form. Whether there is actual multiplication in any part of the digestive tract remains undetermined.

Attempts to acclimate foreign strains of *B. coli* and *Proteus vulgaris* in the intestine of the white rat failed completely. These results are in full accord with those of Seifert (1911) and others.

The results obtained by us on the influence of milk and lactose feeding are in at least partial agreement with the observations of the following investigators.

Weiss (1904) demonstrated the presence of large numbers of *B. acidophilus* in the intestines of persons not suffering with intestinal disturbances of any sort. Since most of these persons had consumed milk previous to the investigation Weiss assumed that the prominence of this organism bears some direct relation to milk diet. Lembke (1897) had shown that a marked difference may be brought about in the character of the intestinal flora by the substitution of a bread for a meat diet, though no prominence is given to organisms of the acidophilic type.

Herter and Kendall (1909) found that the intestinal flora of kittens and monkeys underwent a distinct change when the diet was changed from meat and eggs to milk and glucose. There was a substitution in the feces of an acidophilic type of bacteria for a flora that had been strongly proteolytic. They also observed a marked decrease in the intestine of indol, skatol, phenol and bound hydrogen sulphide, and of the indican and aromatic oxy-acids in the urine. In the light of our own experiments, it appears quite probable that the change in the nature of the flora was brought about by the milk, rather than by the glucose. Friedenwald and Leitz (1909) held that regulation of diet is the most logical way of controlling the intestinal bacteria. De Gaspari (1911) claimed that rats kept on a bread and grain diet showed a predominance of *B. bifidus* over *B. coli*, while meat favored *B. coli* and *B. welchii* to such an extent as almost to exclude *B. bifidus*.

The inhibitive influence of certain sugars and of milk on putrefaction has been known for many years. Hirschler (1886) appears to have been the first to demonstrate such action by carbohydrates. He showed that in vitro the common putrefactive products, indol, phenol, oxy-acids, etc., are held in check in a protein medium if cane sugar, lactose, dextrine, starch or glycerine is added. In the animal body the same results were obtained, but to a lesser degree, owing to the absorption of the carbohydrates from the intestine. The feces of dogs that were fed glycerine, sucrose and starch, together with 250 grams of meat, contained less indol than the control animals.

Rovighi (1892) found that a kephyr diet caused a great reduction in the ethereal sulphates of the urine, and of intestinal indol. He believed that acids played the important rôle of suppressing intestinal putrefaction, but could not substantiate this by practical experiment. Winternitz (1892) demonstrated that milk strongly inhibits putrefaction, and held that this was due to the lactose, and not to the acids resulting from its decomposition. The ethereal sulphates of the urine were reduced and there were other marked indications of lessened putrefaction.

Schmitz (1893) brought about a great reduction in the ethereal sulphates by feeding lactose. He believed, however, that the casein was the important factor in the milk and kephyr. The interesting observations of Bienstock (1901) and others that putrefaction of milk is prevented by lactose-decomposing bacteria are too well known to need comment; as are the repeated demonstrations that sugars are protein sparers in the presence of sugar-fermenting bacteria.

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COCCIDIA IN SUBEPITHELIAL INFECTIONS OF THE INTESTINES OF BIRDS

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In an article entitled "Aberrant intestinal protozoan parasitism" Dr. Theobald Smith (1916) has recently called attention to the presence of a subepithelial infection in a young turkey with a protozoan parasite the nature of which was not definitely ascertained. The presence of a contiguous infection of the intestinal epithelium with a species of coccidium suggested the possibility of the identity of the two forms, but Dr. Smith "is inclined to regard these epithelial cell parasites as belonging to a species distinct from that in the subepithelial tissues. . . . ." The question is raised, however, regarding the frequency of a subepithelial invasion among coccidia, and Dr. Smith suggests the possibility that such invasion might precede the earliest symptoms of the disease and might easily be overlooked.

For several years the present writer has been studying, when opportunity presented, the pathology of avian coccidiosis. The work is still in progress but since some time may elapse before publication it seems desirable to mention in a preliminary way the bearing of certain of the observations upon the problem brought forward by Dr. Smith.

In 1911, the writer (Hadley, 1911) described in detail the process of merozoite formation by the common coccidium of poultry, *Eimeria avium.* It was then stated that the "merozoite cysts" appeared to be of two kinds—one smaller and usually found in the epithelial cells of the upper intestine, the

[1] Contribution No. 222.

latter much larger, and occurring most frequently in the submucosa of the cecal walls. Dr. Smith's suggestion that such a phenomenon might represent in his case an "aberrant" type of infection has led the writer to a re-examination of material reported on in 1911, and to a closer study of other material from cases of coccidiosis in chicks and turkeys obtained since that time. The subject has assumed a special interest since Dr. Smith states that, in the case examined by him, the variation in the products of division, and the irregularity with respect to chromatin distribution leads him to assume that his subepithelial bodies (merozoite cysts?) were largely degenerating forms. This view, according to Dr. Smith "is supported by the abortive attempts at repeated multiplication within the primary cysts and by the partial disappearance of their contents."

The main point which the present writer wishes to bring out in this note is that, as he reported in 1909 and again in 1911, coccidia of the *Eimeria avium* type may be, and commonly are, found beneath the intestinal epithelium. This may occur sometimes in the duodenum, more frequently in the small intestine, and most commonly in the ceca. Coccidiosis in birds appears first in the majority of cases in the duodenum. If the bird survives this initial infection, the lower small intestine may be invaded; and finally the parasites gain lodgment in the ceca. The mature cysts, which are present in almost all poultry, young or old, if occurring in only small numbers in the cecal content or excrement, will usually be found to have had their origin in the epithelial cells of the duodenum. If they are present in large numbers (twenty to fifty per field with $\frac{1}{12}$ oil immersion and No. 4 ocular), the case is unusually severe and it then frequently happens that the epithelium of the lower small intestine and the ceca as well, have contributed to their formation. In the latter case sections of the cecum may show many crypts containing coccidia in various stages of development.

But with reference to the presence of coccidia in the subepithelial territory it is interesting to observe that they may be present there in large numbers even when the adjacent epithelial layers carry but a slight intracellular infection. The

writer has never, however, observed the presence of coccidia beneath the epithelium of the cecum without the presence of some of the same or other stages in adjacent epithelial cells. In many cases, however, the number of organisms located in the subepithelium is entirely out of proportion to the number in epithelial cells, either showing infection or indicating that they have been torn away from their base.

The stage of *Eimeria avium* most common below the epithelium is the "merozoite cyst," usually packed with ripe or immature merozoites. Less commonly the immature schizonts are observed. Macrogametocytes in which the wall has already begun to thicken are also found, but not often. Apparently the situation favors the development of the schizogonous cycle rather than the sporogonous. The merozoite cysts are frequently 50 to 60μ in length, and usually oval. They probably contain several hundred merozoites which are grouped about one or more bodies of reserve substance (Restkörper). Many of the cysts are certainly intracellular and all may be, although in sections it frequently happens that no limiting membrane or crescentic nucleus can be detected. The enclosing cells when present are undoubtedly endothelial in nature in many instances. In others the nature of the host cell has not been ascertained. Some are manifestly giant cells, the several nuclei of which can be observed pressed into a crescentic body at one side of the enclosed parasite. Even here, however, there is no indication that the parasites have been restrained in their development or have degenerated in any way. It seems probable that in some cases more than one merozoite cyst may occupy the same cell as is the case with epithelial cells in which four or five schizonts may frequently be seen.

The merozoite cysts of the submucosa are usually adjacent to the base of the crypts; less seldom in the core of the villi, although they may sometimes be packed in the core to the exclusion of nearly all other cell structures. In such cases they crowd closely on the basement membrane. They are often packed solidly along the muscular wall or in the muscularis mucosa, forming a definite barrier between the inner muscular layer and the

submucosa. In some cases the examination of transverse sections shows the parasites in this position through more than half of the circumference. At other times they are in isolated groups. They often lie closely packed together in a stroma or network of fibres from which most other cells have disappeared—probably from crowding out.

Judging from the number and size of these merozoite cysts, they are very far from being degeneration forms. The size of the merozoites, of which hundreds may be found in the spaces of the mucosa, is the same as that of the merozoites from the duodenum. They stain in the same way and give every evidence of complete development, whether they lie in the endothelial cells or lie separated from the enclosing cell. There is no indication of an "abortive attempt at multiplication."

Dr. Smith raises the question whether a subepithelial development of the coccidia may represent an early stage of infection. In most of the cases examined by the writer, studied by complete series of smears and sections from duodenum, intestine and ceca, the subepithelial infection cannot be regarded as "preliminary," in the sense that it precedes infection in other parts of the intestinal tract. In the cecum it is often present when the duodenum has already been ravaged by the attacks on the epithelium, and the ceca are beginning to show signs of infection in the epithelial cells. Sometimes much of the cecal epithelium in other regions has already suffered considerably and some of the villi are packed with cysts.

A point of considerable interest lies in the origin and manner of infection of the submucosa with the original merozoites that have formed the merozoite cysts. The fact that such infection is observed to exist behind apparently undamaged epithelial walls and is found more commonly in the submucosa adjacent to the bases of the crypts rather than in the cores of the villi, suggests that the parasites may become located here through the agency of the blood stream, having been taken into the circulation in the greatly damaged portions of the duodenum or perhaps in the cecum itself. Here capillaries are frequently broken into; seldom does a severe coccidial infection take place

without a greater or less haemorrhage into some portion of the intestinal canal.

On the other hand it is not impossible that the infection of the mucosa may take place as a result of merozoites or sporozoites penetrating the basement membrane of the epithelial border. One frequently observes in sections the parasite occupying the innermost end of the epithelial cell—the part ordinarily occupied by the nucleus; and in the same field one finds other schizonts located in the core of the villus. This seems to suggest that the original infecting elements may not always rest after they have entered the epithelial cells, but in many cases push on to the basement end, and may sometimes even penetrate the basement membrane and enter the subepithelial territory beyond.

The fate of these merozoites, apparently imprisoned in the subepithelial region is uncertain. How they are able to enter the cecal lumen and infect other cells is not clear unless we assume that invasion of the epithelial cells may take place from the region of the submucosa as well as from the lumen of the crypts. In view of the present observations on the location of mature merozoites behind the intact epithelial wall, this view must be held as an open possibility in coccidial infections. This phase of the matter, as well as other details of the pathology of coccidial infections will receive further consideration in the complete paper to appear, with full illustrations at a later date. It might be mentioned, however, that dissemination of merozoites in the individual through the medium of the blood stream is also a possibility. The writer has examined many blood samples with the hope of discovering either merozoites or some of the flagellated organisms, presumably *Trichomonas*, which are found predominantly in the liver lesions in blackhead of turkeys and other birds, as previously reported by the present writer and confirmed by others. They have not yet been observed, however, although bodies simulating the merozoites were discovered in one case in heart's blood and in liver smears. These bodies, eventually proved to be stages in the life history of a blood sporozoon, probably *Haemoproteus*, since they also

occurred in blood cells. The spindle shaped bodies were only about four to five microns in length, considerably smaller than the merozoites of *Eimeria avium*. Blood from the portal circulation has not been examined.

Thus, to conclude, although the full significance of the presence of merozoites and of other stages of *Eimeria avium*, in subepithelial regions of the intestines cannot yet be grasped, their frequency of occurrence there and their freedom from all appearances of degenerative changes lead us to assume that this phenomenon marks an ordinary phase of the normal infective process; and that so far as the coccidia are concerned, we must, as the writer pointed out some years ago, abandon the view that they are exclusive parasites of epithelial cells in the sense that they must occupy epithelial cells to complete their normal development.

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A BACTERIAL DISEASE OF THE WRAGG CHERRY

PRELIMINARY NOTE

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During the latter part of June, 1915, the writer received some half-grown, green Wragg cherries, affected with characteristic watery, green spots, which varied in size from tiny specks to irregular splotches involving one-half or more of the cherry. In the more advanced stages, these were slightly sunken, brown to black in color, and watery around the margin.

An examination of the watery tissue showed that bacteria were very abundant, which naturally suggested this group of organisms as a possible cause of the trouble.

Further study has shown that as the disease progresses, the spots increase in size and become sunken, while the tissue involved may shrivel and dry down tight to the pit, so that as the cherry fills out and ripens, one whole side or the end presents a large, sunken, mummified spot, black in the center, with a hard, green border. If many such spots occur, the mature cherry is apt to be very irregular and knotty in outline, and worthless for the market. While this condition is not at all uncommon, we also find many ripe cherries with numerous, hard, sunken, green spots of varying size, a stage, apparently, less advanced than the one just described. As the fruit turns red, new infections appear to be fewer, and the progress of the disease seems to be less rapid; in fact, practically no new lesions can be discovered after the cherries begin to color, and those which appear late on the green cherries are discernible only as small, light discolorations in the ripe fruit.

The stems (peduncles) of the cherries are almost invariably attacked and show the infection by turning black and shriveling. In some cases, they are entirely killed, and the cherries drop

prematurely; again, the movement of food substances and water may be interfered with to such an extent as to dwarf the fruit.

The infection is not confined to the fruit, but involves the leaves and young twigs as well. On the former, there occur numerous, more or less circular, chocolate-brown areas, lighter in the center, from 1 to 7 or 8 mm. in diameter. As the dead tissue in these spots dries, it breaks away from the surrounding green area and ultimately falls out, leaving a hole. As a result of this, badly affected leaves present a typical shothole appearance. Neither premature nor serious defoliation of the trees has been observed to take place.

On the young twigs, we find watery, elliptical, olive-brown discolorations, more or less regular in outline, surrounding the lenticels; these elongate with age and become somewhat sunken and darker in color.

From what has been said thus far concerning the disease, it becomes apparent at once that the symptoms are almost identical with the bacterial disease of the peach and plum, described by Smith, Rorer and Rolfs caused by *Pseudomonas pruni* (*Bacterium pruni*, Smith).

Pure culture isolations on nutrient agar from a varied assortment of diseased cherry tissues have yielded an organism which corresponds essentially to *Pseudomonas pruni*. When reinoculated into suitable cherries, it has produced typical lesions and is, therefore, to be regarded as responsible for the above described disease of the Wragg cherry.

While *Pseudomonas pruni* has been assigned as the cause of a similar trouble on other stone fruits, this is the first time, to the writer's knowledge, that its occurrence has been reported on the cherry, and so far as our present field observations go, the disease has been found only on the Wragg variety, although this statement is only tentative.

Spraying experiments with self-boiled lime-sulphur, conducted during the past spring and summer, reduced the injury to the fruit from 41.4 to 10.2 per cent. Further work along this line is to be carried on during the season of 1917.

A detailed account of the investigation will be published in full later.

A STUDY OF THE DIPHTHEROID GROUP OF ORGANISMS WITH SPECIAL REFERENCE TO THEIR RELATION TO THE STREPTOCOCCI[1]

PART I. CHARACTERISTICS OF A PECULIAR PLEOMORPHIC DIPHTHEROID

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The isolation of a diphtheroid bacillus possessed of unusual morphologic and biologic characteristics not only prompted me to make an intensive study of this strain but also directed my attention to a fundamental consideration of the entire group of diphtheroids, having as an object their systematic arrangement into sub-groups. I examined over a hundred strains of these organisms from different sources, some isolated by myself and others obtained from bacteriologists in this country and abroad, representing most of the peculiar types which have been noted in the literature in recent years; and by excluding those forms which were apparently alike I finally selected forty-five representative strains which form the basis of the present inquiry.

As the principal criteria for this classification I have made use of the complement-fixation and sugar fermentation tests; nevertheless I have simultaneously studied the morphologic and other cultural characters of the strains and insofar as possible correlated them with the immunological and fermentative reactions.

In addition to the classification of the diphtheroid group this study has opened up another problem of coördinate importance, viz., a hitherto unsuspected group-relationship between the diphtheria group as a whole and the streptococci, which is ex-

[1] A thesis for the degree of Doctor of Public Health.

emplified in the unusual strain of diphtheroid bacillus to which I have already referred.

The length of the study necessitates its division into three parts, the first of which occupies itself mainly with the strain in question and its relation to the streptococci while the second division deals with the cultural relations and the classification of the sub-types of the diphtheria group itself, and the third with immunologic data.

SOURCE OF THE CULTURE AND CASE HISTORY

On January 24, 1915, a farmer, Mr. J., aged 23, entered the University Homeopathic Hospital, Ann Arbor, Michigan. He complained of a chronic cough which was quite persistent, and had gradually lost weight for the past year. His previous history was uneventful. Nothing of significance in his occupation could be obtained except that he was occasionally engaged in threshing beans, which is very dusty work. There was no family history of tuberculosis. Physical examination showed increased dullness, voice sounds and fremitus over the entire chest. X-ray examination showed a tumorous mass in the mediastinal space opposite the bronchial bifurcation. The Wassermann and Von Pirquet tests were negative. Repeated sputum examinations were negative. Lung punctures revealed the presence of the diphtheroid bacillus described below. The patient's serum agglutinated the diphtheroid bacillus in dilution of 1–80, as against 1–15 for a mixture of 4 normal sera. The serum fixed complement in amounts of 0.1 cc. as against 0.35 cc. for a mixture of 4 normal sera.

On February 15, the patient suddenly developed cyanosis without apparent cause. His condition gradually grew worse until he died four hours after the cyanosis began. The most noteworthy changes occurred in the lungs and heart. The former were not collapsed. They were of a slaty-grey color and adherent to the chest wall in places. On section their resistance to the passage of the knife was greatly increased, and from the moist cut surface a considerable quantity of a frothy, bloody, thin fluid could be expressed. The cut surface had a

red to reddish-brown color interspersed with grey patches which gave it a mottled appearance. Peppered through the patches at regular intervals were very small, pin point masses of a yellowish white caseous material, which was quite adherent to its matrix. This condition was fairly diffuse throughout the lungs. The bronchial glands were greatly enlarged, and no doubt produced the shadow on the X-ray plate at this point, while their pressure against the bronchi may have caused the chronic cough, although it is well known that any diffuse pulmonary fibrosis is liable to be attended by this symptom.

PATHOLOGY OF LUNG OF CASE J

The most conspicuous change in this lung was the wide spread fibrosis and infiltration of the alveolar walls. The latter change was mostly lymphocytic although there were some endothelial cells filled with blood pigment. There was marked chronic passive congestion with focal exudations of red cells. The alveolar spaces were filled with swollen, edematous cells which had sloughed from their walls, a few of which contained blood pigment.

Both Van Gieson's and Mallory's connective tissue stains showed the fibrosis to be limited mainly to the alveolar walls, from which the respiratory epithelium had desquamated. The Gram-Weigert stain showed no fibrin, but did show colonies of diphtheroid bacilli scattered throughout the areas of fibrosis. Scattered at intervals throughout the fibrous tissue were clusters of round or oval bodies from 5 to 7 microns in diameter which were strongly Gram positive. Fuchsin was also tenaciously retained by these structures. They were very probably the products of cellular degeneration known as Russell's bodies.

BACTERIOLOGY OF STRAIN 1

This strain was isolated February 1, 1915, from the case above mentioned which at first was suspected to be one of empyema or pleuritic effusion. It was seen that no fluid was in the pleural cavity, but the syringe when withdrawn contained a few drops of a turbid fluid which was transferred to a blood-agar slant. In six days a very fine, dew drop growth appeared which

on examination proved to be a pure culture of a diphtheria-like bacillus of the barred type. It measured approximately 0.7 by 3 μ and contained from 2 to 5 bars. It stained well in Loeffler's stain and was strongly Gram-positive. The organism was plated out several times on blood-agar to insure its purity and transplants were put in the ice-chest for future animal experimentation.

Most of the virulence tests were done on animals in May, June and July, and several transplants of the organism, which had never been cultivated as a coccus were carried over until October, when further work could be done on them. Although the organism had been found in apparently pure culture and added precautions were taken in this regard by repeated platings, it was deemed advisable to go still further, to be unequivocally sure that we were not dealing with a mixed culture. Such precautions were made necessary by the very extraordinary character of the biologic reaction, viz., the production of lesions characteristic of streptococci of a moderate grade of virulence, but especially by the astonishing pleomorphism which permitted the organism under certain conditions to grow in long chains of diplococci.

Accordingly six single colonies were removed from a tube whose antecedents had never shown any diplococci. Each of these six colonies was examined and then transplanted on plates and tubes to the eighth generation, with the idea that if the culture did contain a lurking coccus it would surely be left behind. Many of these cultures were first shaken with sand (so as surely to separate the organisms) and then plated out. Transplants were made from colonies of the pure bacillus.

I was then able to take these eighth generations in which no diplococci had appeared since the original isolation, and by making use of an expedient which I shall describe presently was able in all six cases to produce from the bacilli, diplococci or diplo-streptococci. At least they took this form when stained with the simple dyes. The method which I used was as follows: a 1 per cent glucose-veal broth, plus 1.6 to phenolphthalein, with a few drops of sterile human or rabbit serum was used for a

medium. A tube of this broth was planted and placed at 37°C. for twenty-four to thirty-six hours. By this time there was usually some flocculation in the bottom of the tube, indicating a slight growth. The tube was then placed at 25° to 30°C. for a few days. At the end of that time as much of the sediment as possible was removed to 25 to 30 cc. of the same kind of broth with a sterile pipette. In twenty-four to seventy-two hours one found the slightly flocculent growth replaced by a very luxuriant, diffuse, finely granular growth, which almost invariably indicated that the form of the organism had undergone a striking change from a barred bacillus to the coccoid or diplococcus form. Short chains also appeared. If the bacillus had not

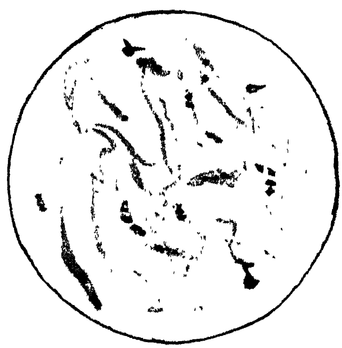

FIG. 1 FILAMENTOUS FORM OF ORGANISM DESCRIBED ABOVE

entirely disintegrated at this time, further transplantation eventually completed the process.

If one cares to examine the flask at four to six hour intervals one can trace the transition from the bacillus to the coccus. It will be seen first, that the chromatin which was distributed irregularly as granules or bars concentrates itself into two masses, and at the same time the body of the bacillus loses its border and becomes striated and indistinct. It stains very poorly. Scattered about the field are irregular masses of débris resembling shreds of mucus. At times this matter forms a peculiar reticulum at whose nodal points will be one or more granules of chromatin. At this time the culture is of a nebulous conglomerate character.

As the culture increases in age the nebulous character gives way to a finely diffuse turbidity while a stringy sediment falls to the bottom of the tube. This sediment is the residue of the disintegrated bacillus, the "shed skin," as it were, while the live organism is suspended for the most part in the supernatant fluid. At this time the supernatant fluid will contain bipolar bacilli with large granules. Their length will vary as will the size of the granules. A definite, faintly staining bond can be seen uniting the two masses of chromatin. In transplants these masses approach each other until the figure cannot be told from a diplococcus, and, what is also of note, these transplants do not contain the débris referred to above. This only appears in the disintegrating stage of the bacillus.

During this metamorphosis a great variety of forms may appear, depending on many factors which one scarcely understands and cannot control. The above picture I have produced with great regularity with many different strains. I cannot produce at will the peculiar reticulated structure, nor can I say with certainty whether the diplococci will be grouped finally like gonococci, staphylococci or streptococci. I have been able with one batch of broth to which I had added a small amount of potassium bichromate solution, to cause these diplococci to grow in long chains, the individual elements of which stained very irregularly by Gram's or Loeffler's methods. With other broth, the same bichromate solution would either fail to do this or might develop bacillary forms from the diplococci.

These various changes in form are to be interpreted as responses of a sensitive organism to a medium of varying chemical constitution. As no two batches of media are precisely alike it can readily be seen that no formula could be laid down for the production of the various forms. However, these changes can be brought about by any one who will take a given medium and modify it in various ways until he accomplishes his purpose. But there are too many chemical factors in media that cannot be held constant for one to attempt to formulate these changes in terms of the physical and chemical constitution of the medium.

We are just beginning to know how to obtain something like a constant reaction in a medium by the method of hydrogen ion concentration.

Much more uncertain and more tantalizing is the conversion of the diplococcus form to the barred, bacillary type. This has been accomplished in various ways. First by an aerobic growth on a partly desiccated blood-agar medium, and second by growth on media containing varying amounts of acid. The change has taken place when the acid concentration has been sufficient almost to prevent growth. Likewise an excess of alkali has also brought about the morphological transformation. These methods may be combined. Simple as these procedures sound I had worked with this organism for nearly a year before I caused it to regain its barred and granular forms, when once it had assumed the streptococcus form. Such transformations were controlled by mmune serum. But here again I know too little about the various factors at play to formulate a method for bringing about this pleomorphism. Anyone repeating this particular procedure must at least be prepared for an exercise of all his patience. I have succeeded in causing the coccoid bodies of *B. Hodgkinii* to regain their bacillary form in one transplant to Loeffler's blood serum. This has also been done by Steele (1914) and others. This procedure was entirely inadequate when applied to strain 1.

Unless great care is used in handling the recently recovered bacillary form, it will almost immediately revert to the diplococcus form when transplanted even to solid media. In this connection I was able to make use of a "relatively acid-fast procedure" (mentioned below) as a demonstration of early change in the bacterial form. I planted a pure culture of the bacillus on blood-agar and developed a grey, moist growth of moderate intensity after twelve hours. Touching the wire very lightly to the surface of the growth and transferring quickly to a cover glass and smearing but once, I disturbed but slightly the orientations of the individual bacilli in the tube. I then stained in carbol-fuchsin and decolorized in a very weak acid-alcohol solution, (1 per cent HCl in 20 per cent ethyl alcohol) washed in

tap water and counter stained in a weak Loeffler's methylene-blue. It will be seen that this strain in the bacillary form stains strongly acid-fast by this method; but the diplococcus form will not hold the carbol-fuchsin under these conditions.

The stained slide showed predominately an intermediate form between the diphtheroid and a coccus although other forms were present. The chromatin bars have formed into two masses (early diplococci) which are roughly wedged shaped resembling *B. Hoffmanii.* The body of the bacillus has "sloughed off" and forms a red staining matrix in which the blue coccoids lie. It is also interesting that quadrants or halves of these otherwise blue wedges will stain a bright red which is really a part of the

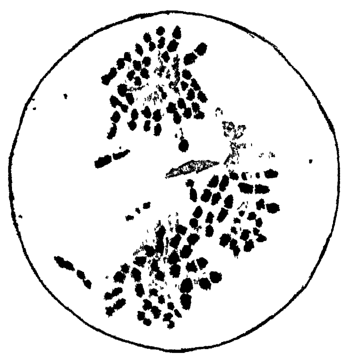

FIG. 2 DISINTEGRATION OF BACILLARY FORM. ORIGIN OF DIPLOCOCCOID FORM

matrix and indicates an imperfect formation of the cocci. The more isolated forms have a narrow, red staining, ragged, irregular rim about them which is nothing but the protoplasmic remains.

When stained by Gram somewhat the same picture is seen. The diplococcus is Gram-positive and thin while Gram-negative strands can be seen extending between the two halves of the organism. Small, Gram-negative spicules project from the bodies of some of the cocci which are merely the remains of the partly disintegrated protoplasm.

The organism is non-motile and does not form spores. Capsules have been demonstrated in the bodies of animals. It is a

facultative anaerobe. It has the intense viability characteristic of the whole group, and in this respect more than in any other can this strain be said to differ from the streptococci. It is extremely resistant to desiccation and to the addition of antiseptic substances to the medium. Its thermal death point is 58° to 60°C. in three minutes. It grows at room temperature under favorable conditions, and long exposure to sun light does not kill it.

It does not liquefy gelatin but the growth in this medium is very good. The bacillary form of the organism has fastidious cultural characteristics, while the diplococcoid form is very adaptable. Animal fluids with the exception of ascitic fluid facilitate its growth. In this medium multiplication is quite slow. The bacillary form is well preserved in ascitic-broth with a marked tendency to form branching and fusing involution forms. All media give better results with the addition of 1 per cent glucose. The amount of acid formed in glucose broth is not sufficient to kill the organism after seven to ten days at 37°C. This is not usually true with the streptococci. In common with the other members of the diphtheroid group this organism attacks the protein of the medium after the available sugar has been fermented, giving rise then to an alkaline reaction.

On blood-agar slants the bacillary form develops very fine transparent colonies in from twenty-four to forty-eight hours, after which they become translucent or grey. In case the slant is kept moist by turning the condensation water over it, the colonies often become opaque and increase greatly in size. Microscopic examination at this time reveals nothing but cocci and coccoids. A luxuriant, coalescing, moist growth on any medium is always an indication of cocci and coccoids, while a granular discrete translucent or transparent growth is characteristic of the bacillary forms.

In glucose-broth the bacillus forms a sparse granular growth which settles to the bottom of the tube or clings to its walls. It may become flocculent or nebulous in character depending largely on the presence of serum in the medium. If the

supernatant becomes diffusely turbid, it is indicative of the formation of coccoids and cocci. The organism grows well in litmus milk, reddening the litmus in twenty-four hours and producing marked coagulation in from forty-eight to seventy-two hours. No gas is formed. It grows fairly well at room temperature. No indol is produced and only a trace of nitrites. No growth occurs on potato. Glycerin, glucose, lactose, maltose, sucrose, dextrin, inulin and salicin are fermented with acid but no gas formation.

TOXIN FORMATION

The following charted curves are suggestive of the action of a soluble toxin.

Chart I shows the weight and temperature curves of rabbit 3. On the vertical axis is registered the weight of the animal in grams, the curve of which is represented by a solid line. The lower part of this axis also registers the temperature in degrees Fahrenheit, the curve of which is represented by a broken line. The horizontal axis registers the dates on which readings were made while the injections are shown at *x* and *xx*.

Chart II reproduces the essential parts of the protocol of rabbit 4. These experiments were undertaken with the view to determining the ability of the organism studied to yield a soluble toxin. The luxuriantly growing diplococcus form of the bacillus was planted in 1 per cent glucose-broth for twenty-four hours, after which it was filtered through a Berkefeld W filter and tested for its sterility. The sterile filtrate was then sealed and placed in the ice-chest.

On October 20 and 23, and November 4, rabbit 3 received 1 cc. of this filtrate and on December 14, it received intravenously 5 cc. of a very heavy emulsion of a twenty-four hour growth on an agar slant. It is seen that the animal show an immediate response to the filtrate injection by a sudden and progressive loss in weight which remains constant the first time at 1435 grams. Another injection at this point not only fails to decrease the weight further, but does not prevent it from returning to its former level. Since the interval between

the second and third injection is quite appreciable this may be accounted for on an immunity basis. It may also be accounted for on the theory of the degeneration of the toxin to toxoid as the

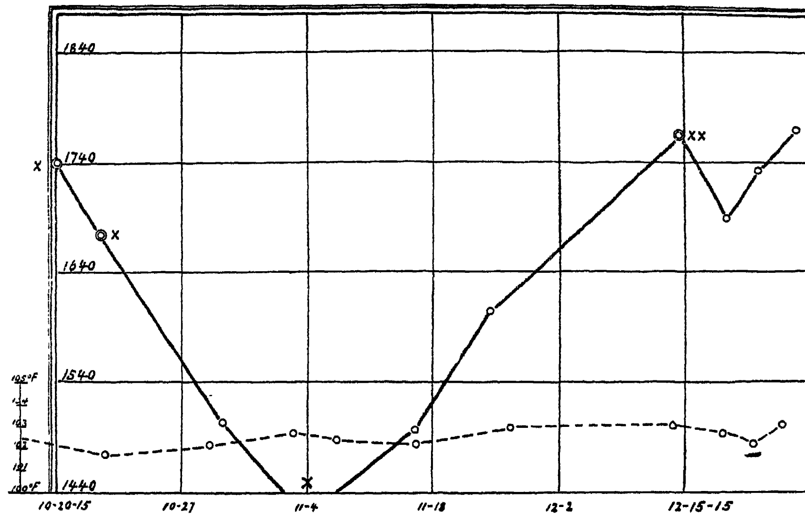

CHART I. RABBIT 3

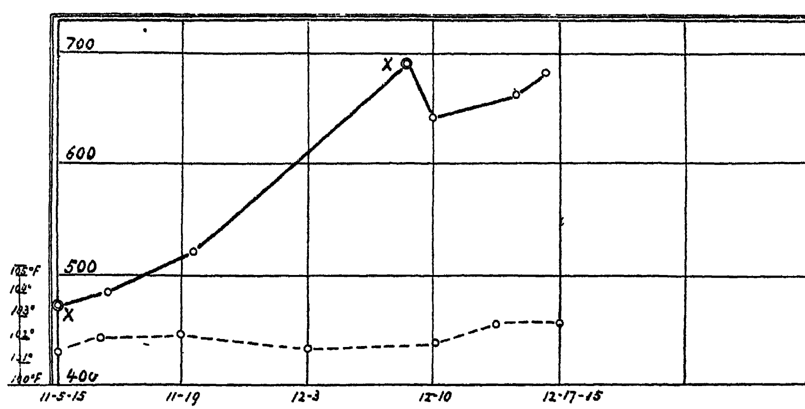

CHART II. RABBIT 4 (CONTROL TO RABBIT 3)

result of standing in the ice-chest. It is interesting that the injection intravenously of an enormous dose of the live culture in an animal thus protected caused only a transient loss in weight.

Although the culture had lost some of its virulence at this time, parallel injections of such large doses in normal animals were always attended with serious results. It is quite noteworthy that at no time did the temperature curve rise above normal. It varied between 102°F. and 103°F. irrespective of the injections. This experiment gives suggestive evidence of the mild toxicity and corresponding protecting action of the germ-free broth filtrate.

On November 4, rabbit 4 was given an intraperitoneal injection of the identical filtrate received by rabbit 3 which had stood in the ice-chest since October 20, 1915. This experiment with a very small rabbit was undertaken for the purpose of determining whether the toxin had degenerated, and if so, whether the toxoid also had protective action. It will be seen by the curve that the animal continued to gain weight gradually, the same as did rabbit 3, after the synchronous injection, all of which is evidence of the degeneration of the substance present in the fresh filtrates. An intravenous injection of 1 cc. of a heavy suspension of a twenty-four hour agar culture produced about the same effect as in rabbit 3. The fact that the weight loss was very transient and no temperature rise was produced, offers additional evidence for the above hypothesis. Rabbit 5 of the same weight as rabbit 3 was treated in the same way and gave the same results, so its curve will not be shown.

In looking over the protocols of a large number of rabbits injected intravenously, I find that they always develop a rise in temperature when the live culture is given. This rise averages about 2.5° to 3° above the mean temperature of the rabbit used.

I wish in this connection to insert a very interesting protocol of an animal which had received several injections of heat killed salt suspensions of this strain for immunization purposes. It had developed no agglutinin in the serum following any of these injections but a week after an injection of the live organisms the titre was about 1–200.

The injections in doses of 1, 1, 1.5 and 3 cc. were given on October 16, 23, and November 9 and 15 respectively. The weight was markedly affected, as with the germ-free filtrate,

while the temperature remained constant. On December 1, the rabbit received intravenously 3 cc. of a heavy suspension of a twenty-four live agar culture. The weight remained constant, while there was a sharp temperature rise, just as in untreated animals. This experiment forms a very convincing control on the temperature criterion and serves to illustrate the idea that the killed organisms apparently protect against the cachexia, although permitting a temperature rise with live or-

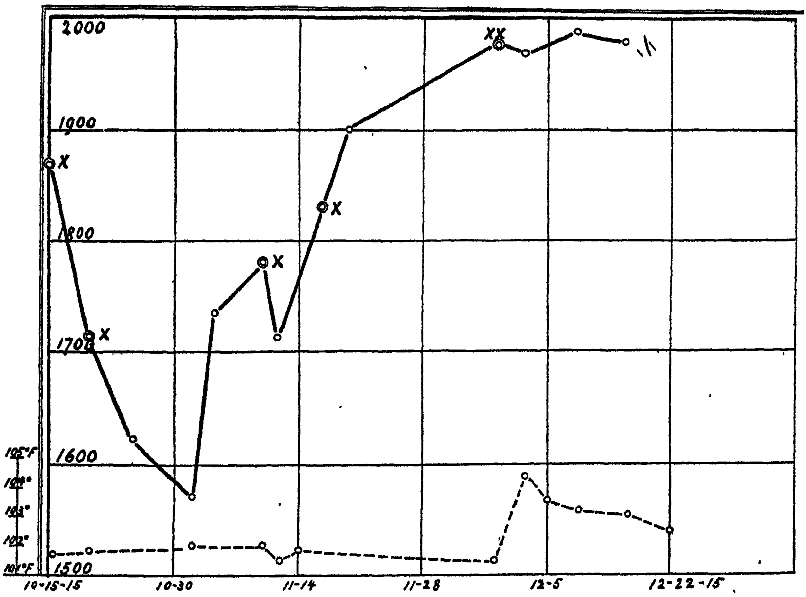

CHART III. RABBIT 1

ganisms, while previous injections of the toxic filtrate prevent both the cachexia and the rise of temperature.

Attempts were made to develop active protective power by use of the germ-free filtrate. The following charted protocols are typical of the results obtained.

The double *xx* indicates an injection intravenously of live organisms while the single *x* indicates filtrates.

Rabbit 6 (Chart V) received subcutaneously 1.5 cc. of a week old toxic filtrate on November 13, 1915. On November 16 and 19, it received intraperitoneally 2.5 and 4 cc. respectively, on Novem-

ber 18, 1915, 2 cc. intravenously. After one of these injections only, was any reaction shown. On November 16, within two minutes following the injection the rabbit developed a mild clonic convulsion interrupted by short periods (five to ten seconds)

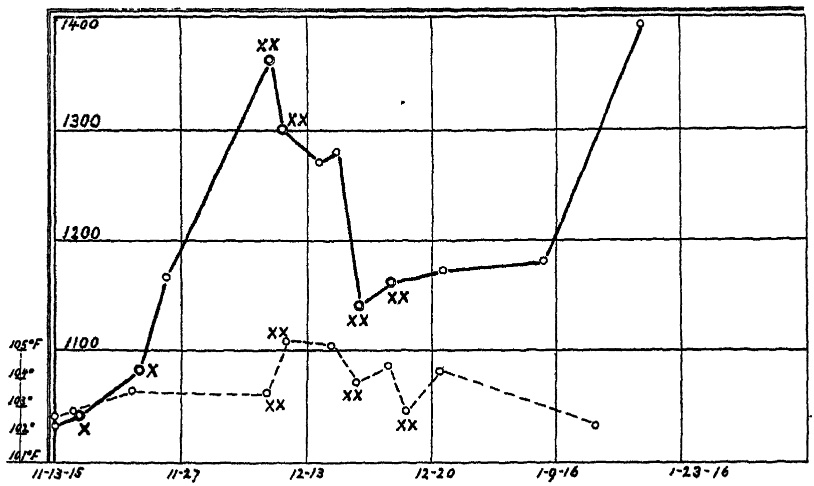

CHART IV. RABBIT 7

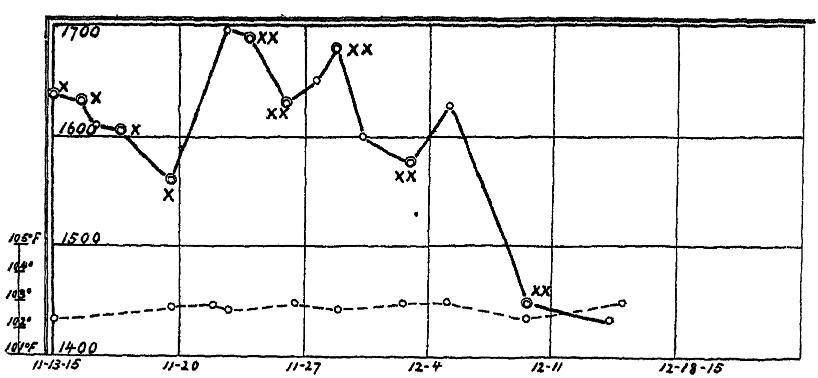

CHART V. RABBIT 6 (CONTROL TO RABBIT 7)

in which the fore- and hindlegs were extended to their limit. Reflexes were much increased. When the skin was touched the animal developed a generalized tremor. Involuntary evacuation of urine and feces occurred. The paroxysm lasted from

three to five minutes, after which the animal seemed normal except for a generalized muscle fibrillation. The temperature was subnormal (100°F.) for thirty minutes after the injection.

On December 4, 11, 15, 23, and January 7, the animal received respectively 1.5 cc., 1.75 cc., 2 cc. and 3 cc. intravenously of a salt suspension of a twenty-four hour culture on agar. The loss in weight was progressive for the first two weeks while the animal received the filtrate. Its weight was then held well until after the third injection which forced it slightly below normal, but after the fourth injection the drop was quite marked. It is again noteworthy that there was no temperature rise at any time which is but a repetition of the experiment with rabbit 4. This curve shows how the filtrate protects against the cachexia but this protection is limited, since multiple injections of the live organism ultimately break down whatever immunity has been formed. This experiment also serves as a control to rabbit 7.

On November 13, 1915, rabbit 7 (Chart IV) received intraperitoneally a mixture of 1.5 cc. filtrate plus 0.5 cc. of the antiserum of rabbit 3. On November 16, 1915, it received 2.5 cc. filtrate plus 1 cc. serum of rabbit 3 (intraperitoneally). No paroxysm nor subnormal temperature followed this injection as in rabbit 6. As the curve shows, the animal gained weight rapidly and consistently during the next three weeks. On December 7, 11, 15, and 23 it received intravenously 1.25 cc., 1.5 cc., 2 cc., and 2.5 cc. respectively of a live salt suspension of a twenty-four hour agar slant culture. In contradistinction to No. 6 every injection was followed by a rise in temperature of 2 to 2.5°. Likewise there was a rapid and progressive diminution in weight. When the injections were stopped the weight was gradually restored and the temperature soon receded. This animal which was given a neutralized toxin (or toxoid) showed the same reactions as an untreated animal except perhaps that its weight recovery after cessation of the injections was more prompt.

Further experiments of similar nature were undertaken to find out if possible the nature of the toxic substance in the filtrate. Unfortunately the bacillus which by this time had been subcultured through many generations had not only undergone

a perceptible diminution in virulence but had completely lost the power of producing a toxic filtrate, at least when ordinary doses of it were injected. Attempts were made to recover its toxic production by employing the most favorable means for the development of true diphtheria toxin. A rich veal broth with 0.1 glucose was placed in flasks affording the best aerobic conditions. This was incubated one week and filtered, but the results were negative.

When the toxic substance was present in the filtrate, it was found that heating to 85°C. for one-half hour rendered it harmless, i.e., no weight loss followed its injection. The effect of heating was the same as that which took place spontaneously when standing in the ice-chest.

To reiterate: This strain produced in broth cultures after twenty-four to forty-eight hours growth a soluble poison which resembled in many ways a true toxin. It did not react vigorously as most true toxins do. It was altered rather rapidly in the ice-chest and was destroyed by heat. Intraperitoneal injection of both the fresh product and the toxoid conferred a protective power against its characteristic effects. An injection of the serum of a treated animal when mixed with the toxic filtrate was attended by no symptoms. The organism lost this power of producing a poisonous filtrate after eight months of cultivation on artificial media. The toxin differed materially from true diphtheria toxin in the fact that toxin-antitoxin mixtures failed to protect.

In this connection the observation of Thiercelin on the enterococcus is very interesting. This organism is one of the most pleomorphic known and has a very remarkable cultural and biological similarity to the strain which I have described. The following quotation from Besson (1913) is noteworthy.

The saprophytic enterococcus though incapable of infecting the rabbit will nevertheless often kill the animal by toxemia. Following an injection of culture the animal sickens and becomes cachextic, develops paralytic symptoms and dies on an average in a fortnight to twenty-five days. Subcutaneous inoculation of a culture of the saprophyte filtered through a Chamberlain bougie leads to the death

of the rabbit from cachexia. (Thierceln and Jouhaud): The same result has been obtained with cultures which have been sterilized by boiling for thirty minutes or by heating to 110°C. for fifteen minutes.

Biologically this toxic product resembles very closely what Ehrlich describes as the toxon of the Klebs-Loeffler broth filtrates. He says (Zinsser 1908) that

> These toxons which he regards as primary secretion products of the bacilli, possess a haptophore group identical with that of the toxin but have a different toxophore group. For there is reason to believe that the toxon, lacking the power of causing acute death, gives rise to slow emaciation and paralysis finally killing after a subacute or chronic course.

On account of the close relationship of strain 1 to the streptococcus group, the production of similar substances in filtrates is interesting. Roger (Besson 1913) grew a streptococcus anaerobically in meat broth at 30°C. for five days, and obtained a germ-free filtrate product which caused cachexia and wasting and finally death of the animal. Injection of the unheated culture in sub-lethal doses predisposed the animal to infection with the homologous strain; but after heating to 104°C. the culture had some protective value. He concludes that there is a thermostabile substance present as well as a destructive thermolabile substance. This resembles somewhat the action of the enterococcus filtrate.

PATHOLOGIC ANALYSIS

Reference to table 1 shows several important facts. First, that certain organs are affected to the exclusion of others, irrespective of the dose employed; second, a general progressive diminution in the virulence of the organism; third, that without regard to whether the bacillus, diplococcus, coccoids or mixed forms are injected the lesions are the same; and fourth, that even though the pure bacillus may be injected, yet from some organs the diplococcus form will be the predominating variety recovered. Although as I shall show later on, this phenomenon is an example of extreme pleomorphism, nevertheless the relation

to the streptococcus group suggested by this radical morphological departure is entirely vindicated by the pathologic and immunologic results produced.

The organism has almost a coördinate predilection for the gall bladder and joints, although when the virulence is much attenuated the joints seem to be no longer affected (rabbit 2). The skeletal muscles are next most prominently affected while acute nephritis and congestion are inconstant. The lesion in the gall bladder is that of acute catarrhal cholecystitis. In the joints there is an acute suppurative arthritis. The muscles show acute degenerative myositis, infarction and calcification. The latter is the most prominent change. The lymphocytic infiltrative change is not marked. The predominating renal change is severe cortical congestion with some acute parenchymatous nephritis. In one animal there were two small medullary abscesses produced. In the abdomen of animal 43 there was noticed a clot of blood about 6 mm. in diameter. Sections showed that most of the blood was peripheral while in the center was lymphatic and pancreatic tissue. In this area were found endothelial-celled thromboses in the small lymphatics and in the veins. The extravasation was also marked around these vessels.

TABLE 1

Pathological conditions produced by intravenous injection of strain 1 isolated from case J

ANIMAL NO.	DATE OF INJECTION	FORM OF BACTERIA	TOTAL DOSAGE	AUTOPSY*	LESIONS IN Appendix	Stomach duodenum	Gall bladder	Pancreas	Joints	Endocardium	Skeletal muscles	Kidney	Lung	Skin
			cc.											
40	May 14, 21, 25, 28, 31	Diplococcus form of diphtheroid	7.5	June 2	0	–	+++ s†	0	++c	0	0	+c	0	0
46	May 14, 15, 16	Pure barred bacilli	7	May 20	0	0	+++bs	0	++s	0	++b	+c	0	0
47	May 14, 21	Pure barred bacilli	10.0	May 24	0		+++cs	0	++bc	0	0	++b	0	0
48	May 15, 16	Mixed cocci and bacilli	15.0	Dead May 20	0	+c	+++s	0	+++cs	0	+++cs	++	0	0
41‡	June 5, 6, 8	Cocci	11.0	Dead June 9	0	+c	+++s	0	++cs	0	++cs	++c	0 0	0
42	June 5, 6, 8	Mixed cocci and bacilli	10.0	June 10	0	+	+++s	0	++sc	0	+++bc			
43	July 5, 6	Bacilli	4.5	July 15	0	Congestion	+++s	+ c	+++cs	0	+c	++c	0	0
45	June 11, 15	Mixed cocci and coccoids	30.0 Sediment	June 30	0	0	++cb	0	++cb	0	+cb	+c	0	0
2	Oct. 16, 26, Dec. 1	Cocci	5.5	Dec. 3 Dead	0	0	++cb	0	Non-purulent exudation	0	0	+++c	0	Diffuse congestion
49	Feb. 15, 17, 18	Cocci	8.0		0	0	0	0	0	0	0	0	0	0

* Unless otherwise indicated the animal was chloroformed.

† The letter c following a + indicating organ involvement means that the diplococcus form of the bacillus was recovered from that organ. Letter s means the streptococcus, and the letter b indicates recovery of the bacillary form.

‡ This animal received on June 8, 1.5 cc. of the Lewis streptococcus the action of which simulates strongly that of strain 1.

DETAILS OF EXPERIMENTAL PATHOLOGY

Rabbit 40

The organism with which this animal was injected had grown for some weeks on blood-agar following a determination of its purity. It had been converted into a diplococcus after a method described on page 84. All the injected diplococci were prepared in this way.

On May 14, 1915; May 21, 25, 28, and 31, this animal received 1.5 cc. of a glucose serum broth culture of these diplococci, making in all 7.5 cc. The reaction of the broth used was +0.15 to 0.2 to phenolphthalein. A few drops of serum were put in a tube to enrich the medium. In this time the animal lost weight falling from 1525 grams to 990 grams. Temperature rose 2°. Animal chloroformed June 2, and autopsied immediately.

Protocol. Small amount of cloudy peritoneal fluid. Kidneys and liver show severe congestion. Lungs and heart negative. Free clots and fluid in pleural cavity. Congestion and enlargement of lymphatic glands throughout. Suppurative arthritis. Gall bladder filled with turbid hemorrhagic fluid.

Smear. Heart blood: leucocytosis and lymphocytosis. Diplococci with moderate phagocytosis. Liver same. Spleen: few polymorphs but many splenocytes and lymphocytes. Small amount of phagocytosis noticeable. Kidney: only an occasional polymorph showing ingested organisms. Peritoneal fluid: many polymorphs with marked phagocytosis. Joints: same. Cultures from all the above organs (except the mesenteric glands) showed diplococci or streptococci.

Rabbit 46

On May 14, 15, 16, it received 2, 2.5, and 3.5 cc. of thick NaCl suspension from a blood-agar slant intravenously. Etherized May 20. Positive changes were as follows: gall bladder reddened and distended. Contains turbid hemorrhagic bile. Joints had viscid exudate, kidney showed marked congestion. Intercostal and abdominal muscles showed diffuse patches of myositis. These areas were whitened and translucent or opaque. From these areas were isolated pure barred bacilli while the joints and bile contained streptococci and the kidney diplococci. Cultures in broth were all positive.

Rabbit 47

On both May 14 and 21, the animal received 5 cc. of NaCl suspension of bacilli intravenously. The results were the same as in rabbit 46.

Rabbit 48

On May 15 and 16, the animal received intravenously 5 and 10 cc. of glucose-serum-broth culture of diplococci and bacilli mixed. The lesions produced were exactly the same as in rabbit 47 except two or three small hemorrhages in the gastric mucosa. The gall bladder contained long chains of streptococci and clumps of bacilli.

Rabbit 41

On June 5, 6, and 8, the animal received 5 cc., 5 cc., and 1 cc. respectively of serum-broth-culture of diplococci intravenously. On June 8, the injection consisted of genuine streptococci. Found dead in the cage on June 9. Autopsy immediately. No rigor. General lymphadenitis with regional congestion of efferent vessels. Two small hemorrhagic spots over right lower thorax. Many ecchymoses, especially on thorax. Extravasation in loose subcutaneous tissue over lower thoracic and lumbar vertebrae. Extended to muscular aponeurosis. Heart and various organs yielded cultures of cocci in pure culture or mixed with a short barred diphtheroid. Gall bladder, joints, skeletal muscles and kidneys all involved while stomach showed four pin-point hemorrhages.

Rabbit 43

On July 5 and 6, this animal received intravenously 2 and 2.5 cc. respectively of bacilli grown in glucose-broth. Was very stupid on July 8, 1915. Seemed to have a general tremor. Temperature was 41°C. From July 5 to July 15, it lost weight from 1390 to 940 grams. Temperature did not go above 41°C.

Protocol. Skin and superficial lymphatics negative. Small areas of myositis in brachialis, latissimus dorsi, and intercostal muscles. Liver congested. Kidneys seem enlarged. Cortex was congested while on one of the collecting pyramids were seen two small abscesses about 2 to 4 micra in diameter. Organ as a whole rather pale. Diffuse congestion of stomach near pylorus; no hemorrhage. Bile ex-

tremely viscid and contained fine floccules. Heart and lungs negative. Joints contained thick viscid exudate.

Smears of the joints showed a marked acute purulent synovitis. The exudate contained many agglomerations of coccoids and diplococci imbedded in a faintly or negative staining matrix; some short chained streptococci; encapsulated diploccoci. Bile contained many desquamated cells and an enormous number of very long chained streptococci which showed a most remarkable morphologic variation. A single chain contained lanceolate forms, diplococci, and rows of granules simulating the streptococcus form of the diphtheroid bacillus. There were present all gradations morphologically between a coccus and a bacillus (see Plate 2).

A broth culture of the bile stained in Gram showed about the same picture except that the bacillary forms were wanting. It is quite noteworthy that the chains, individually and collectively, showed all grades of reaction to the Gram stain. Many were Gram-positive, but at one or more points in the chain one could see the blue granules which made the entire chain resemble a thread of diphtheroid bacilli. Further subcultures developed diplo-streptococci with the Loeffler stain, but with Gram the irregular granular character persisted.

Immediately below the stomach in the region of the pancreas was seen an irregularly shaped body about 6 mm. in diameter. It apparently was a blood clot. It was cultured and then put in fixing fluid. The most conspicuous pathological lesion found was an endothelial-celled thrombosis (Plate 1). It was interesting that the weight loss in most rabbits was marked while the temperature reaction was only moderate.

Rabbits 42 and 45

These rabbits showed no lesion which did not appear in the others and it is not worth while to give a detail of the protocol.

Rabbit 2

On October 16, and 26, this animal was given intravenous injections of 0.05 and 1 cc. respectively of serum-broth cultures of diplococci. It decreased in weight from 1000 grams to 915 grams on October 27, and the temperature did not go above 103.5°F. From November 1, the animal gained steadily in weight until December 1, when it weighed 1360 grams. It was then given one half of a blood-agar slant of diplococci (twenty-four hour culture) which killed it in twenty-four hours.

Postmortem. There was a diffuse redness of the subcutaneous tissue and a very severe acute nephritis. The medulla of the kidney was especially involved. Bile was also turbid, flocculent and hemorrhagic. The joints had a very small amount of nonpurulent exudate. The heart-blood and bile showed many diplococci by culture in glucose-broth but the joints were negative. It is rather noteworthy that this is the only instance in which the joints did not show a purulent exudate and result positively in the culture.

Rabbit 49

This animal was given intravenous injections of the diplococci on February 15, 17, and 18, 1916 in doses of 2 cc., 3 cc., and 3 cc., respectively. There was only a weight loss of 250 grams in a 1500 gram rabbit after one week. By March 1, this was regained and the animal has been well ever since and on April 1, weighed 1800 grams. Evidently the culture had lost its virulence to a marked degree. This feature has been quite noticeable in the experiments.

RESEMBLANCE TO STREPTOCOCCI

This organism has apparently an elective affinity for the gall bladder, the joints, and skeletal muscles primarily, and for the kidney and stomach secondarily. It is noteworthy that this localization compares well with that of the vast majority of strains of streptococci of moderate or low virulence (Rosenow 1915 a). Since this organism is not only capable of taking on the form of the streptococcus and producing similar sugar reactions but also of causing almost identical lesions, it is highly probable that biochemically there is a relation between these two groups. Accordingly my immunization work (to be discussed later) had as one of its chief aims the detection of cross reactions between the diphtheroid and streptococcus groups.

RELATION OF STRAIN 1 TO THE ENTEROCOCCUS

I have already referred to the similarity of action between the filterable poisons of these two organisms. That there is a further general parallelism between them becomes very apparent on studying the original description given by Thierceln (1903) and Besson (1913).

The enterococcus is one of the most pleomorphic organisms known, and the detailed description of Thierceln (1903) gives most of the various forms which I have described. However he does not speak of its resemblance to the diphtheria bacilli. The cultural characteristics with the exception of the sugar reactions are practically identical. Besson speaks of its poor growth in milk and of the fact that it does not usually coagulate. Strain 1 coagulated milk very decisively by fermenting lactose. The authors above mentioned make no further reference to sugar reactions either negatively or positively. Park and Williams (1910) speak of it as not fermenting sugars, nor producing indol or odor in the presence of sugar.

It has produced in both man and animals multiple suppurative arthritis, pleuritis, post-typhoid suppuration, enteritis, hepatitis, meningitis and pseudo-lobar broncho-pneumonia. These features make it resemble a streptococcus closely. Indeed Eserich, Tavel, and Eguet have described encapsulated streptococci in the intestines of new born children and in 1894–7 Besson described a "new encapsulated streptococcus" which he had isolated from two cases of post-typhoid suppuration. (Purulent pleurisy, multiple suppurative arthritis.) These organisms are identical with the enterococcus. Its wide distribution and prolonged vitality are also points in common with strain 1.

RELATION TO ERYTHEMA NODOSUM STRAIN

Rosenow (1915 b) has described a diphtheroid which he has shown to be the cause of erythema nodosum that also bears a striking similarity to strain 1. He describes it as a Gram staining, polymorphic non-motile, non-spore-bearing bacillus producing small round colonies in glucose-agar, and small grey or yellowish, non-hemolyzing colonies on blood agar, and having a wide range of fermentative power. He says of his strain 929:

All gradations in form between straight and clubbed bacilli, some of which retained Gram's stain while others did not, and Gram-positive

elongated diplococci, and perfectly round coccus forms were found in each of the ten colonies examined. The aerobic cultures of Loeffler's-serum and blood-agar showed a predominance of coccus forms often in short chains in place of bacilli.

On planting the same colony in different media he obtains bacillary forms from one culture and pure diplococcal forms from the other. Again

In three cases it was quite impossible to decide whether the organism isolated should be regarded as a streptococcus with marked involution forms or as a diphtheroid bacillus with streptococcal forms. In no instance was there a mixed infection with a bacillus and a streptococcus. On cultivation in some of the media and on injection into animals, streptococcal forms were produced freely.

SUMMARY

Strain 1 of my series is representative of a sub-group of diphtheroids which has received scant recognition in the literature. Its pleomorphism is rivaled by that of the enterococcus to which it is closely related. The so-called *B. Hodgkinii* although very pleomorphic does not show the protean morphology of this strain. I feel that the evidence regarding its causal relation with the unique pulmonary condition from which it was isolated is adequate. The fact that it was isolated from the lung in pure culture several weeks before the patient died, and that his serum gave positive agglutinin and complement-fixation reactions is very suggestive. Demonstration of the bacillus in the colonies imbedded in the increased connective tissue of the alveolar walls, the lack of fibrin plugs, indicating pneumococcal infection, the absence of the tubercle bacillus or its tissue lesions, the lack of dust in sufficient amount to give rise to the condition, form with the serum reactions quite convincing evidence of causal relationship.

Finally the pathogenicity of this organism for laboratory animals, and its close relation to the streptococcus group amplifies the facts above given. Had the organism been injected into animals immediately after it was isolated, the chances for

developing pulmonary lesions would have been much greater, as Rosenow has convincingly shown in the case of the streptococcus (1915 a). The very fact that it was 'nothing but a diphtheroid' relegated the culture to the ice-chest until a convenient season arose for its testing.

I have already described some of the cardinal characteristics of this strain in The Medical Record (Mellon, 1916) and have pointed out its probable relation to the streptococci. The erythema nodosum strain also belongs to this group as well as others in the series later described in this study. The immunological reactions, of both agglutinins and complement-fixing bodies are further evidence for the same contention. Not only morphologically and culturally, but biologically as well this strain has much in common with the streptococci; and a thorough study demonstrates that it represents a group of organisms standing in an intermediate position between the diphtheroid and the streptococcus groups.

(To be continued)

Note. After this paper had gone to press there appeared a description of a new type of a diphtheroid organism by Walker and Adkinson (1917) resembling closely the bacillus whose characters are depicted here. It appears that they had overlooked my original brief description of this organism (1916) and in a personal communication from Dr. Walker he expresses the opinion that the organisms are identical.

EXPLANATION OF PLATES

Plate 1

1. Case J. Low power view of lung showing diffuse interstitial fibrosis and small round-celled infiltration.
2. High power view of the same.
3. High power of no. 1. Marked focal infiltration and fibrosis of alveolar wall.
4. Desquamated edematous respiratory epithelium lying free in the alveolar space.

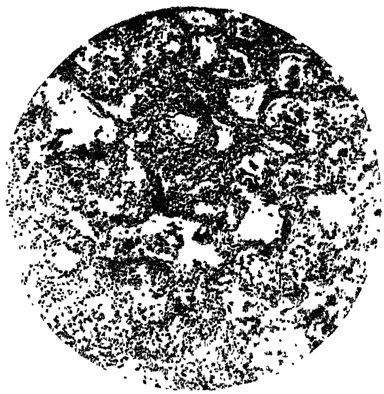

FIG. 1

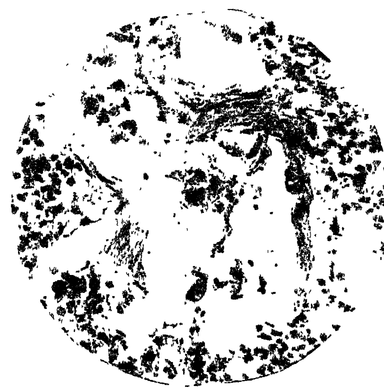

FIG. 2

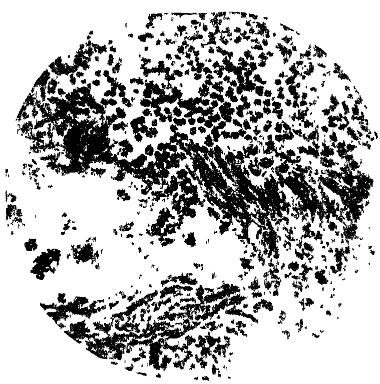

FIG. 3

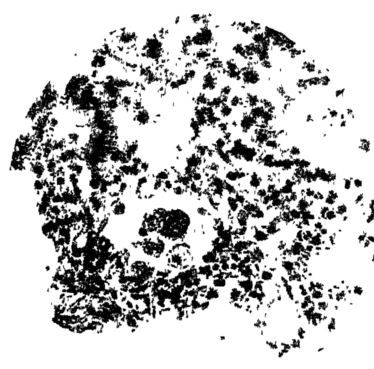

FIG. 4

(Mellon: Diphtheroid Group of Organisms)

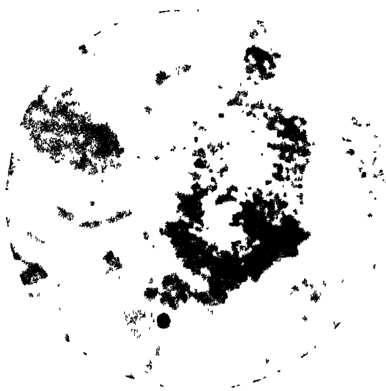

Fig. 5

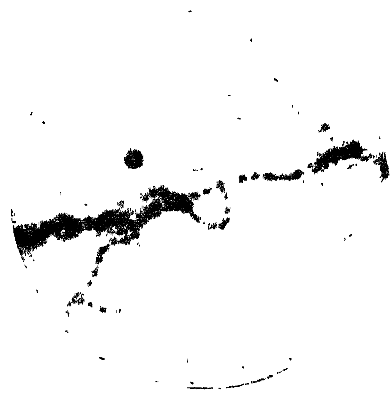

Fig. 6

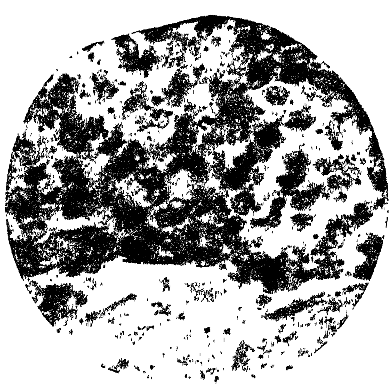

Fig. 7

Fig. 8

(Mellon: Diphtheroid Group of Organisms)

PLATE 2

5. A colony of diphtheroid bacilli lying in the interstitial tissue of the lung. Gram stain.

6. Smear from cholecystitis in rabbit 43 showing morphologically a streptococcus. The bacillary form of the organism was injected intravenously.

7. Endothelial-celled venous thrombosis in a rabbit (43) caused by injection of strain number I, case J.

8. Myositis in latissimus dorsi muscle of the same rabbit. Notice areas of calcification and early fibrosis with but slight infiltration.

THE COLORIMETRIC DETERMINATION OF HYDROGEN ION CONCENTRATION AND ITS APPLICATIONS IN BACTERIOLOGY

PART II

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SECTION VIII. THEORY OF INDICATORS

This is not a proper place for a detailed discussion of the theory of indicators.[13] For adequate treatment of the manifold aspects of the subject the special literature must be consulted. In the papers of Noyes (1910) and Bjerrum (1915) will be found discussions and references to the literature bearing upon many of the theoretical aspects of the present discussion. The treatment in these papers has been developed chiefly with reference to the theory of titration, and it may therefore be profitable to review very briefly a few of the more important principles involved in this other use of indicators in order that those not familiar with the subject may gain an orderly and concise view of the logic of the colorimetric method, and in order that certain methods of expressing ideas which we wish to emphasize in a later discussion may be clear.

According to the theory of electrolytic dissociation an acid of the type HA dissociates as follows:

$$\mathrm{HA} \rightleftarrows \underset{\text{cation}}{\overset{+}{\mathrm{H}}} + \underset{\text{anion}}{\overset{-}{\mathrm{A}}}$$

[13] A detailed discussion is not necessary because the colorimetric method is to a very large extent a *comparative* method with hydrogen electrode measurements as the basis. Neither dissociation constants nor theories in regard to the nature or seat of color changes enter into the practical use of indicators in determining hydrogen ion concentrations. Plant pigments of unknown constitution have been successfully used.

The equilibrium of this reversible reaction is expressed in accordance with the mass law in equation (1).

$$\frac{[H] \times [A]}{[HA]} = K \tag{1}$$

Here [H] is the concentration of the hydrogen ions, [A] the concentration of the anions, [HA] the concentration of the undissociated residue, and K is a constant, which, although it depends upon the temperature and the nature of the solvent, is characteristic of a given compound under set conditions. K is termed the dissociation constant. If equation (1) is written in the following form,

$$\frac{[A]}{[HA]} = \frac{K}{[H]}$$

it is readily seen that the ratio of the anions to the undissociated residue is determined by the dissociation constant of the acid and by the hydrogen ion concentration of the solution. If now we represent the concentration of the total acid in whatever form by S, then the concentration of the undissociated residue is S–[A]. Hence:

$$\frac{[A]}{S - [A]} = \frac{K}{[H]} \quad \text{Or} \quad \frac{[A]}{S} = \frac{K}{K + [H]}$$

$\frac{[A]}{S}$ is the ratio of the anions to totally available acid. This ratio may be represented by α when the equation becomes:

$$\alpha = \frac{K}{K + [H]} \tag{2}$$

Were we to plot α against [H] we should obtain a hyperbolic curve difficult to handle, but if, as Henderson (1908), Sörensen (1912), Michaelis (1914) and others have done, we plot α against P_H, we obtain the form of curve shown in figure 5. In this figure the abscissas are P_H values and the ordinates α values expressed as percentage dissociation. The value of K (equation 2) determines the position of each of the curves. When K

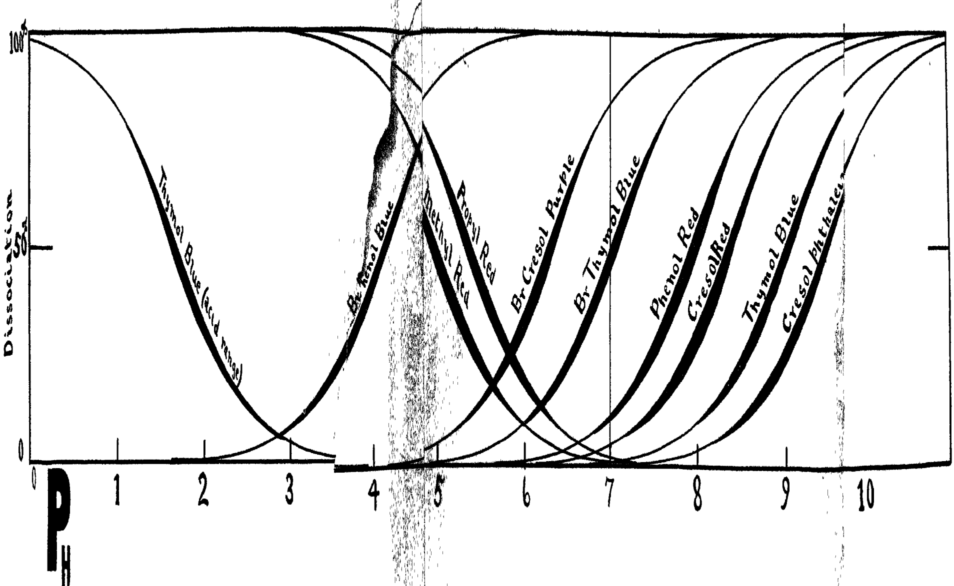


FIG. 5. Dissociation curves of indicators considered as simple mono-acids, showing percentage color change with P_H. Shaded portions of curves indicate the useful ranges.

= [H] equation (2) reduces to $\alpha = \frac{1}{2}$, or, in other words, when the P_H is such that the corresponding hydrogen ion concentration is numerically equal to the dissociation constant, the acid is half dissociated.

It will be evident without developing separate equations that the curve showing the percentage of undissociated residue at each P_H is the complement of that showing the percentage dissociation and has the form of the curve for methyl red in figure 5.

In a similar way the equations and curves showing the percentage dissociation of a base at different hydroxyl ion concentrations may be developed. Such an equation is

$$\alpha = \frac{K_b}{K_b + [OH]} \tag{3}$$

Since we wish to deal only with [H] we must obtain [OH] in terms of [H]. $\frac{[H] \times [OH]}{[H_2O]} = k$. Since $[H_2O]$ may be considered a constant we may write the above equation $[H] \times [OH] = K_w$ Whence $[OH] = \frac{K_w}{[H]}$

Substituting this in equation (3) we have

$$\alpha = \frac{K_b}{K_b + \frac{K_w}{[H]}} \quad \text{or} \quad \alpha = \frac{K_b \times [H]}{K_b \times [H] + K_w}$$

The undissociated residue is $1 - \alpha$. Now

$$1 - \alpha = 1 - \frac{K_b[H]}{K_b[H] + K_w} \quad \text{or} \quad 1 - \alpha = \frac{K_w}{K_b[H] + K_w} \tag{4}$$

If we do not know whether we are dealing with an acid with dissociation constant K_a or a base with dissociation constant K_b, K_b may be related to K_a as follows; $K_b = \frac{K_w}{K_a}$. If we substitute this in equation (4) we obtain

$$1 - \alpha = \frac{K_a}{[H] + K_a} \tag{5}$$

The right hand side of equation (5) is identical with that of equation (2). In other words the curve for the undissociated residue of a base is identical with the curve for the dissociation of an acid when the acid and basic dissociation constants are related as $K_b = \frac{K_w}{K_a}$. In a similar way it may be shown that when these dissociation constants are thus related the curve for the dissociated portion of a base is identical with the curve for the undissociated residue of an acid. Thus we cannot tell by the form or the position of such curves as are shown in figure 5 whether we are dealing with an acid or a base. We cannot tell by the conduct of methyl red for instance whether we are dealing with an acid with acid dissociation constant 1×10^{-5} or with a base with basic dissociation constant $\frac{1 \times 10^{-14}}{1 \times 10^{-5}} = 1 \times 10^{-9}$.

For the decision we must turn to chemical evidence.

Now let us assume, as Ostwald (1891) did, that indicators are acids or bases whose undissociated molecules have a different color from that of their dissociation products. It will then be readily seen that the percentage color of indicator solutions at different P_H values may be shown by the curves in figure 5. If we consider for the sake of simplicity of treatment that phenol red is a simple acid of the type HA and that the undissociated HA group is yellow while A is red then we may represent the percentage of the dominant red in solutions of this indicator at different P_H values by the dissociation curve shown in figure 5. The dominant red of methyl red we may represent by the curve marked "methyl red" and this may be either the curve of the undissociated residue of an acid or that of the dissociated portion of a base.

In fixing the positions of these curves we have had to determine K the apparent dissociation constant of each indicator. This was found by the following method. If, for example, we add ten drops of a phenol red solution to 10 cc. of a buffer solution of such a P_H value that the indicator is half transformed, we may regard the equivalent of five drops as transformed to the red form and the equivalent of the other five drops as exist-

ing in the yellow form. We should then be able to duplicate the color of this mixture by superimposing 10 cc. of a very alkaline solution containing five drops of the indicator, which will be fully transformed into the red form, upon 10 cc. of an acid solution containing five drops of the yellow form. To gain equal depths of solutions through which to view the colors we may arrange them as follows.

alkaline solution (red) 5 drops indicator	known P_H standard 10 drops indicator
acid solution (yellow) 5 drops indicator	water

Now the P_H *of the upper right hand solution* is varied until a color match with the above arrangement is observed. It is then assumed that the P_H of the solution we have been varying causes a half transformation of the indicator. As has been shown on page 111 the corresponding [H] is equivalent to the dissociation constant of the indicator.

This method was first used by Salm (1906). It will be recognized as a crude method in many respects, but it enables us to determine the dissociation constants with sufficient accuracy for the present purposes of illustration. The values so obtained are given in table 3.

With the aid of the approximately determined apparent dissociation constants we are enabled to plot the curves shown in figure 5 which reveal graphically the relationships of the various indicators in the series we shall discuss. This figure shows at a glance that an indicator of the simple type we have assumed has no appreciable dissociation and consequently exists in only one colored form at P_H values beginning about 1.5 point below

TABLE 3

Approximate apparent dissociation constants of indicators

INDICATOR	K	P_H
Phenol phthalein	2.0×10^{-10}	9.7*
o-Cresol phthalein	4.0×10^{-10}	9.4
Carvacrol sulfon phthalein	1.0×10^{-9}	9.0
Thymol sulfon phthalein	1.2×10^{-9}	8.9
α-naphthol phthalein	4.0×10^{-9}	8.4
o-Cresol sulfon phthalein	5.0×10^{-9}	8.3
α-naphthol sulfon phthalein	5.3×10^{-9}	8.2
Phenol sulfon phthalein	1.2×10^{-8}	7.9
Dibromo thymol sulfon phthalein	1.0×10^{-7}	7.0
Dibromo o-cresol sulfon phthalein	5.0×10^{-7}	6.3
Dipropyl red	4.0×10^{-6}	5.4
Dimethyl red	7.9×10^{-6}	5.1†
Tetrabromo phenol sulfon phthalein	7.9×10^{-5}	4.1
Thymol sulfon phthalein (acid change)	2.0×10^{-2}	1.7

*This value is identical with Rosenstein's (1912).

†In the table published in the Journal of the Washington Academy, Vol. vi, p. 485, these values for methyl red and propyl red were erroneously interchanged. Tizard (1910) gives $K_A = 1.05 \times 10^{-5}$ or $P_H = 4.98$ for methyl red considered as an acid.

the half transformation point, while at the same distance above this point the indicator is completely dissociated and exists only in its second form. Between these limits the color changes may be observed. The useful range of such an indicator is far less than 3 points of P_H and for the following reasons. In the high dilutions in which solubility limits and other considerations force us to use indicators no distinct color change will be observed until a considerable degree of dissociation occurs. We must not only be able to detect the colors, we must also be able to distinguish the differences at adjacent P_H values. In order that this may be accurately done the color intensities must be well beyond the "threshold" for the eye and the percentage increase in color in the indicator with change in P_H must be large. In short, distinct intensities or differences in color must appear before they can be distinguished by the eye. On the other hand as the indicator reaches a higher degree of dissociation, each increment becomes a smaller and smaller percentage of the whole

and the eye is unable to distinguish the differences amid the intense coloration.

These and other factors limit the range over which any one indicator may be used. Accurate determination of the limits would be a research problem in itself. We have attempted, however, to show the approximate range of usefulness by means of the shaded portion of each of the curves. This will indicate that the limits assigned in the tables are not rigid but may be extended beyond the most useful range when necessary.

The illustration (fig. 5) will show how in choosing a set of indicators it is advantageous to include a sufficient number, if reliable indicators can be found, so that their ranges overlap. It shows that each of the indicators, when considered to be of the simple type we have assumed, has an equal range. It also shows that the half transformation point of each indicator occurs nearer one end of the useful range.

It is evident that if the actual color change of an indicator varied with P_H in accordance with a curve such as those in figure 5, and if the true dissociation constant were accurately known, then the hydrogen ion concentration of a solution could be determined by finding the percentage transformation induced in the indicator. Indeed the dissociation constants of some few indicators have been determined with sufficient accuracy to permit the use of this method when the proper means of determining the color intensities are used. Such use of indicators may be made independent of hydrogen electrode measurements. But for reasons which will presently become evident this procedure is impracticable at present.

We have been assuming that the theory of indicators may be treated in the simple manner originally outlined by Ostwald (1891). In his theory it was assumed that the anion of an indicator acid, for instance, has a color different from that of the undissociated molecule. This assumption if unmodified does not harmonize with what is known. Researches in the phenomena of tautomerism have shown that when a change in color is observed in an indicator solution the change is associated with the formation of a new substance which is generally a

molecular rearrangement or so-called "tautomer" of the old. If this color change is associated with the transformation of one substance into another, how is it that it seems to be controlled by the hydrogen ion concentration of the solution? As Steiglitz (1903) and others have pointed out, it is the state of these compounds, their existence in a dissociated or undissociated condition, which determines the stability of any one form. In other words it is, after all, the degree of dissociation, as determined by the hydrogen ion concentration, which determines which tautomer predominates. Therefore, consideration of the tautomeric equilibria only modifies the original Ostwald treatment to this extent; the true dissociation constant is a function of the several equilibrium and ionization constants involving the different tautomers and must be replaced by what Acree calls the "total affinity constant" or by what Noyes calls the "apparent dissociation constant," when it is desired to show directly how the color depends upon the hydrogen ion concentration.

Many indicators are poly-acidic or poly-basic and will not rigidly conform to the treatment for a simple mono-basic acid such as we have described. Phenolphthalein, for instance, as was shown by Acree (1908) and by Wegscheider (1908) must be considered as a poly-basic acid. The proper equations to apply in this case have been given by Acree (1907, 1908) and also by Wegscheider (1908, 1915). According to Acree and his students (Acree, 1908) (Acree and Slagle, 1909) the chief color change in phenol phthalein is associated with the presence of a quinone group and with the ionization of one ofthe phenol groups. In the sulfon phthalein series of indicators Acree and his students (White 1915 and White and Acree 1915) have found much the same sort of condition. In the sulfon phthalein series, however, certain unique properties, which will be further described by Lubs and Acree in a paper soon to be published,[14] make the series eminently suited for experimental demonstration of the seat of color change. We may mention here that in the case of those sulfon-phthalein indicators with low apparent dissocia-

[14] This paper by Lubs and Acree has now appeared in *J. Am. Chem. Soc.* 1916, **38**, 2772.

tion constants these constants are so low and the dissociation constants of the sulfonic acid groups are so high that we may without any serious error treat these compounds, so far as their color transformations are concerned, as if they were simple mono-basic acids. As in the case of thymol blue the two sets of color transformations are so far apart on the P_H scale that they do not interfere.

The second set of color transformations which is observed with thymol blue in very acid solutions (low P_H) we have treated *as if* they were connected with an electrolytic dissociation as they apparently are. Without any regard for the nature of this transformation we have determined the apparent dissociation constant in the manner previously described and have constructed the curve shown in figure 5. The transformation of thymol blue in acid solutions makes it useful in exactly the range which the curve indicates.

These curves, while they have been constructed for purposes of illustration only and have been based on the simple and somewhat incomplete treatment described, illustrate with greater clarity and in more detail the useful ranges of the indicators than would a mere tabulation of these ranges. The ranges found with the aid of these curves are found to be consistent with those empirically established.

Figure 5 may also be used in a later discussion to illustrate the relation of P_H to the dissociation of acids and bases.

SECTION IX. OPTICAL ASPECTS

While the color changes of indicators are correlated with molecular rearrangements controlled by hydrogen ion concentrations, it should not be forgotten that the phenomena observed are optical and that no theory of indicators can be considered complete enough for practical purposes which fails to recognize this. As ordinarily observed in laboratory vessels, the color observed is due to a somewhat complex set of phenomena. It is unfortunate that we have no adequate treatment of the subject which at the same time embraces electrolytic dissociation, tautomerism and the optical phenomena in a manner directly

available in the practical application of indicators. The simultaneous treatment of these various aspects is necessary before we can feel quite sure of our ground when dealing with the discrepancies often observed in the comparison of colorimetric and electrometric measurements of biological fluids.

There are many solutions so turbid with suspended matter or so rich in color that accurate measurement of their hydrogen ion concentrations by means of indicators is almost out of the question. The obscuring effect of the "natural color" of culture media has been one of the greatest obstacles to the application of the colorimetric method. Sörensen, however, has said of the natural color of solutions that it does not produce the confusion that might be expected. Our own experience corroborates this. Nevertheless, both the color of culture media and the suspensions of cells, precipitated peptones, etc., which are found in active cultures, are serious embarrassments to be dealt with, carefully when possible, and sometimes by bold methods.

There have been two chief methods of dealing with the interfering effect of the color of solutions. The first method, used by Sörensen (1909 a and b) and adequately described by him, consists in coloring the standard comparison solutions until their color matches that of the solution to be tested, and subsequently adding to each the indicator. In many cases, culture media have a yellow appearance which can be approximately matched by one of the indicators. The yellow form of methyl red does very well for alkaline solutions and that of phenolsulfonphthalein (phenol red) for the acid solutions. In no case, however, can the matching be made perfect even with the use of an elaborate set of colors and in most cases it is a troublesome process.

The second method was introduced by Walpole (1910). It consists in superimposing a tube of the colored solution over the standard comparison solution to which the indicator is added, and comparing this combination with the tested solution plus indicator superimposed upon a tube of clear water. A convenient instrument for this purpose is now on the market. At

the time our researches were undertaken, we were unable to get the Walpole instrument, and we therefore used a homemade "comparator" similar to that described by Hurwitz, Meyer and Ostenberg (1916). It consists of a block of wood with holes bored to receive four test tubes. Holes are made in the block so that these test tubes can be viewed from the side in pairs. One pair is: tube of solution plus indicator, tube of clear water. The other pair is: tube of comparison solution plus indicator, tube of solution. This is the Walpole combination. The device is optically very imperfect but it works fairly well. When we speak subsequently of the use of a "comparator" we mean this device.

One or another of the means described serves fairly well in overcoming the confusing influence of moderate color in solutions to be tested. In bacteriological work, however, a most serious difficulty is presented by the suspension of cells and precipitates.

If one views lengthwise a tube containing suspended particles, or even particles of colloid dimensions, much of the light incident at the bottom is absorbed or reflected before it reaches the eye, and, if the tube is not screened, some of the light which reaches the eye is that which has entered from the side and has been scattered. Consequently, a comparison with a clear standard is inadequate.

Sörensen (1909 a and b) has attempted to correct for this effect by the use of a finely divided precipitate suspended in the comparison solution. This he accomplishes by forming a precipitate of $BaSO_4$ through the addition of chemically equivalent quantities of $BaCl_2$ and Na_2SO_4. Strictly speaking, this gives an imperfect imitation, but like the attempt to match color it does very well in many instances. The Walpole superposition method may be used with turbid solutions as well as with colored, as our experience with the device of Hurwitz, Meyer and Ostenberg has shown. In passing, attention should be called to the fact that the view of a turbid solution should be made through a relatively thin layer. When the comparison is made in test tubes, for instance, the view should be from the side. There are some solutions, however, which are so dark or

turbid that they can not be studied by any of the methods so far mentioned. On the other hand, certain very dark solutions such as the darker bouillons, and potato juice oxidized to an apparently *black* solution, we have handled very successfully by the dilution method which will be discussed in the section on approximate procedures.

It is obvious that, whether the interference is due to color or turbidity, brilliancy of an indicator will aid greatly in overcoming it. Furthermore, the "color change" of a one-colored indicator like phenolphthalein or paranitrophenol is to a large extent a difference of intensity without any noticeable change in quality. The color change of a two-colored indicator, on the other hand, is a change in quality which up to a certain limit is unmistakable even when turbidity or other colors interfere. Brilliant two-color indicators are therefore, from the subjective point of view, preferable. It is for this reason that we prefer the brilliant two-color indicators of the sulfonphthalein series.

We feel sure that those who use the sulfonphthalein series of indicators, which we are describing in this paper, will be impressed by the advantage of their wonderful brilliancy. This, combined with their relatively small protein and salt errors, makes the series eminently useful. We must, however, mention in this section a phenomenon, which undoubtedly is exhibited to some extent by solutions of all of these compounds, but which becomes so prominent in certain cases that it may produce confusion or errors if not recognized. The phenomenon we speak of is the dichromatism exhibited, for instance, by solutions of brom phenol blue. Solutions of this indicator appear blue when viewed in thin layers but red in deep layers. The explanation is as follows: The dominant absorption band of the alkaline solution is in the yellow and the green, so that the transmitted light is composed almost entirely of the red and blue. The incident light has an intensity which we may call I. After transmission through unit thickness of solution some of the light has been absorbed and the intensity becomes Ia, where a is a fraction—the transmission coefficient—which depends upon the nature of the absorbing medium and the wave length of the

light. After traversing thickness $^{\epsilon}$ the intensity becomes $I\,a^{\epsilon}$. Now the transmitted blue is $I_b a_b^{\epsilon}$ and the transmitted red $I_r a_r^{\epsilon}$. We do not happen to know what the actual values are, but let us assume first that the intensity of the incident blue is 100 and of the red 30 and that $a_b = 0.5$ and $a_r = 0.8$.

For $\epsilon = 1$, $I_b a_b^{\epsilon} = 50$ and $I_r a_r^{\epsilon} = 24$. Hence blue greater than red.

For $\epsilon = 10$, $I_b a_b^{\epsilon} = 0.01$ and $I_r a_r^{\epsilon} = 0.30$. Hence blue less than red.

This example indicates that the solution may appear blue when viewed through thin layers while it may appear red when viewed through thick layers.

If we change the relative intensities of the incident red and blue we can change the color of a given thickness of solution. If in the above example we reversed the intensities of the incident red and blue, then,

For $\epsilon = 1$, $I_b a_b^{\epsilon} = 15$ and $I_r a_r^{\epsilon} = 80$ or red greater than blue.

This is essentially what happens when we carry the solution from daylight, rich in blue, to the light of an electric carbon filament lamp, poor in blue. The solution which appears blue in daylight appears red in the electric light.

The practical importance of recognizing the nature of this phenomenon may be illustrated in the following way. Suppose we have a solution rich in suspended material, such as bacterial cells, and that we wish to determine its P_H value by using brom phenol blue. If we view such a solution in deep layers very little of the light incident at the bottom reaches the eye. A large proportion of the light which does reach the eye is that which has entered from the side, has been reflected by the suspended particles, and has traversed only a relatively thin section of the solution. In such a solution then, if it is of the proper P_H, brom phenol blue will appear blue, while in a clear comparison solution of the same P_H the indicator appears red or purple if the tube is viewed lengthwise. A comparison is therefore im-

possible under these conditions. If, however, we view the two solutions in relatively thin layers, as from the side of a test tube, they will appear more nearly comparable. There will still remain, however, a clearly recognizable difference in the quality of the color which serves as a warning that the two solutions are not being compared under proper conditions. We can obtain the proper conditions only when we eliminate from the source of light either the red or the blue, so that the phenomenon of dichromatism will not appear. Which had best be eliminated is a question which can not be answered properly until we have before us the necessary spectrometric measurements. Nevertheless the following observations made with a small hand spectroscope, and the deductions therefrom may prove to be illuminating.

The chief absorption bands of brom phenol blue solutions occur in the yellow-green range and in the blue. In alkaline solutions the band in the blue disappears while that in the yellow widens into the green. As the solution is made more acid the band in the blue appears, shutting off the transmitted blue, while that in the yellow-green contracts, permitting the passage of the green. Our light source then should be such that at least one of these changes may become apparent, and at the same time either the blue or red must be eliminated. The light of the mercury arc fulfills these conditions. It is relatively poor in red and it emits yellow, green and blue lines where the shifts in the absorption bands of brom phenol blue occur. Since the mercury arc is not generally available we have devised a light source to fulfill the alternative conditions, namely, one which will permit observation of the contrasts due to the shift in the yellow-green band[15] and which at the same time is free from blue. Such a source is found in electric light from which the blue is screened by a translucent paper painted with an acid solution of phenol red. The arrangement we have used is described in the section on apparatus. One disadvantage of

[15] This should not be confused with the changes in "subjective color." In the screened light no participation of transmitted green will be detected by the unaided eye.

such a screen is that the red transmitted through it is so dominant that it obscures the contrasts which are due to the shifting of the yellow-green absorption band. Nevertheless, such a screen has proved useful in P_H determinations with brom phenol blue and particularly useful with brom cresol purple. In either case it is most useful in the more acid ranges covered by each of these indicators.

While considering light sources we may call attention to the fact that all the sulfonphthalein indicators may be used in electric light, although brom thymol blue and thymol blue are not well adapted for use in light poor in blue. Doubtless a more thorough investigation of the absorption spectra of the sulfonphthalein indicators will make it possible to devise light sources which will materially increase their efficiency.

So far as we have been able to detect with instruments at hand, the absorption spectra of all the indicators of the sulfonphthalein series are such that the appearance of dichromatism must be expected under certain conditions. It will be observed with phenol red in light relatively poor in red and rich in blue, for example, the light of a mercury arc; and with thymol blue in light relatively poor in blue and rich in red for example, ordinary electric light.

It may be noted that many colored culture media absorb blue light strongly and that this may be connected in some way with the slight errors frequently noted in P_H determinations with the blue indicators.

SECTION X. PROTEIN AND SALT ERRORS

In the correlation of electrometric and colorimetric measurements discrepancies have often been traced so clearly to two definite sources of error that they have been given categorical distinction. They are the so-called "protein" and "salt" errors.

From what has already been said in previous pages, it will be seen that if there are present in a tested solution bodies which remove the indicator or its ions from the field of action either by absorption or otherwise, the equilibria which have formed the basis of our treatment will be disturbed. An indicator in

such a solution may show a color intensity, or even a quality of color, which is different from that of the same concentration of the indicator in a solution of the same hydrogen ion concentration where no such disturbance occurs. We could easily be led to attribute very different hydrogen ion concentrations to the two solutions. This situation is not uncommon. The most striking instance which we ourselves have observed is the precipitation of congo red upon the surfaces of curd grains and, presumably, the absorption of this indicator by the casein in milk. Effects with similar results but with indefinitely known causes occur very generally when native proteins or some of their products of hydrolysis are present in solution or suspension. Such effects when attributable to protein or even peptone are classed in the category of "protein errors."

If two solutions, each containing the same concentration of hydrogen ions, are tested with an indicator, we should expect the same color to appear. If, however, these two solutions have different concentrations of salt, it may happen that the indicator color is not the same in both solutions. As Sörensen (1909) and Sörensen and Palitzsch (1913) have demonstrated, this effect of the salt content of a solution cannot be tested, as Michaelis and Rona (1909 a) at first supposed, by adding the salt to one of two solutions which have previously been brought to the same hydrogen ion concentration. The added salt, no matter if it is a perfectly neutral salt, will change the hydrogen ion concentration of the solution to which it is added. The influence of salts is felt then alike by indicators and by the constituents of buffer mixtures. The nature of this influence is at present so little understood that it cannot yet be treated in a systematic manner. Harned (1915) has shown that salts exert specific effects upon the hydrogen ion concentration of KOH solutions and Kolthoff (1916), has found specific effects in the action of different salts upon indicators.

A model of the manner in which the salt errors may be estimated and the proper corrections applied in specific cases is found in the work of Sörensen and Palitzsch (cf. Sörensen and Palitzsch, 1913, and other papers) upon the hydrogen ion con-

centration of sea water. Sea water does not vary greatly in the nature of its salt content. A systematic calibration of indicators when used at different concentrations of the salt water is therefore possible.

In dealing with protein solutions calibration is less certain. When solutions to be tested vary greatly, not only in protein content but also in the composition and concentration of their salt content, systematic calibration becomes very difficult. When there are added the difficulties presented by strong coloration and turbidity, calibration is impossible. Such is the situation to be faced when dealing with the media and the cultures which the bacteriologist must handle. We can bring to bear upon the problem no adequate explanation of the "salt effects," no general theory of the "protein errors," no comprehensive treatment of the optical difficulties, and finally no perfectly rigid basis upon which to compare the electrometric and colorimetric measurements. We have therefore considered it wise to leave any detailed treatment of these subjects to painstaking research upon restricted cases and upon more favorable material.

Such considerations should not deter us from choosing those indicators which give the most consistent values. When the agreement is good in a very wide variety of cases we may safely consider the method reliable for approximate determinations, without seeking to classify small discrepancies which may be observed.

SECTION XI. APPROXIMATE PROCEDURES

There are many instances where accurate determinations are not essential, but where approximate measurements have a distinct value. One instance is to be found in the method of Clark and Lubs (1915) for the differentiation of the two main groups of the colon-aerogenes family of bacteria. In this method the composition of the medium is so adjusted to the metabolic powers of the organisms that the medium is left acid to methyl red by one of the groups and alkaline to methyl red by the other group. In the original description of this test the differentiation was

made by simply adding the indicator and noting the difference in color. With very little additional time and labor determinations of the P_H values may be made. We have made what we consider to be very good approximate measurements with the *B. coli* cultures at the rate of 60–100 an hour by the following procedure. The cultures are grown in 5 cc. portions of the special medium held in uniform 10 cc. test tubes. When the indicator test is made one worker runs in 5 cc. of an aqueous solution of methyl red from an automatic pipette. (The quantity of indicator thus added must of course be adjusted to equal that added to the standard comparison tubes.) A second worker makes the colorimetric comparisons. In this way a definite amount of indicator is added to each tube and a dilution is made which reduces the obscuring effect of the turbidity of the culture.

We have mentioned the dilution method as a means of reducing the coloration and turbidity of solutions to be tested colorimetrically. Dilution will, of course, change the hydrogen ion concentration of a solution, but it can be shown that moderate dilution in most cases does not change the hydrogen ion concentration seriously. The following brief theoretical outline will indicate the reason.

Let us consider an acid of the type HA, for the dissociation of which we have the equilibrium equation:

$$\frac{[H] \times [A]}{[HA]} = K_a \tag{1}$$

If the acid alone is present in the solution we may assume that $[A] = [H]$ Also, if S_a = the total acid, $[HA] = S_a - [H]$. Introducing these into equation (I) and solving for [H] we have

$$[H] = \sqrt{K_a S_a + \frac{K^2_a}{4}} - \frac{1}{2} K_a$$

When K_a is small in relation to S_a

$$[H] \cong \sqrt{K_a S_a} \tag{2}$$

Thus the hydrogen ion concentration [H] varies with dilution (diminution of S_a) of the solution only as the square root of K_aS_a. The special case in which we have to do with a very dilute solution of a practically completely dissociated acid is rarely met with in physiological studies.

Equation (1) may be written

$$[H] = K_a \frac{[HA]}{[A]} \tag{3}$$

If there is present a salt of the acid, this salt may furnish some of the anions [A]. Since salts are generally more strongly dissociated than the acids, [A] may be furnished almost entirely from the salt, if it is relatively sufficiently concentrated and the acid is weakly dissociated. Furthermore, as the dissociation of the acid is suppressed by the high relative concentration of A, [HA], the concentration of the undissociated portion, approaches the molecular concentration of the acid, S_a. In the extreme case where the acid is weak in dissociating power, and the salt of this acid is relatively concentrated, we may represent the equilibrium expressed in equation (3) by equation (4)

$$[H] = K_a \frac{S_{acid}}{S_{salt}} \tag{4}$$

In other words the hydrogen ion concentration varies only as the ratio of acid to salt, K_a being a constant. Since this ratio does not change on dilution the hydrogen ion concentration will not change when the solution is diluted.

This conclusion holds only with the above mentioned simplifying assumptions. Actually the *relative* concentration [A] in the denominator of equation (3) increases when the solution is diluted, because of the increase in the *percentage* dissociation of both acid and salt. Consequently [H] decreases.

Thus, when a mixture of an acid and its salt is diluted, the hydrogen ion concentration varies somewhat more than the zero variation shown by equation (4) but less than that indicated by equation (2). A similar conclusion would be reached if the case of a mixture of a base and its salt were considered. Fur-

thermore the acids and bases thus considered may be regarded as the components of poly-acids or poly-bases or of amphoteric electrolytes.

But changes in [H] seem less when expressed as P_H which is $\log \frac{1}{[H]}$. Thus, to halve the hydrogen ion concentration, P_H must be increased only about 0.3 points. But to accomplish this in the first extreme case mentioned we should have to dilute the solution about four times. If a mixture of acids or bases with their salts is being dealt with the change of P_H on fourfold dilution will be *very* much less.

Sörensen (1912) has given some calculated P_H values for different dilutions of asparagine and glycocoll which are types of the amphoteric electrolytes found in many culture media. His values are as follows:

MOLECULAR CONCENTRATION OF GLYCOCOLL	P_H	MOLECULAR CONCENTRATION OF ASPARAGINE	P_H
1.0	6.089	1.0	2.954
0.1	6.096	0.1	2.973
0.01	6.155	0.01	3.110
0.001	6.413	0.001	3.521
0.0001	6.782	0.0001	4.166

The dilution here is ten-fold at each step, yet the increase in P_H is very small while the solutions are as concentrated as 0.1–0.01 M.

When dealing with complex solutions which are mixtures of very weakly dissociated acids and bases in the presence of their salts, and especially when the solution is already near neutrality dilution has a very small effect on P_H, so that if we are using the crude colorimetric method of determining P_H a five-fold dilution of the solution to be tested will not affect the result through the small change in the actual hydrogen ion concentration. Differences which may be observed are quite likely to be due to change in the protein or salt content. For this reason as well as for other reasons we have considered it wise to use M/20 standard comparison solutions instead of more concentrated standards. The salt content of the standards undoubtedly influ-

ences the indicators and should be made as comparable as is convenient with the salt content of the solutions tested.

The conclusion that dilution has little effect on the hydrogen ion concentrations of many solutions has long been recognized. Michaelis (1914) found little change in the P_H of blood upon dilution, and Levy, Rowntree, and Marriet (1915) have depended upon this *in part* in their dialysis method for the colorimetric determination of the hydrogen ion concentration of blood. Henderson and Palmer (1912) have used the dilution method in determining the P_H of urines, and Paul (1914) records some experiments with wines the P_H values of which were affected but little by dilution. The legitimacy of dilution has been tacitly admitted by bacteriologists in their procedure of diluting media to be titrated to what is in reality a given P_H as indicated by phenolphthalein.

The dilution proceedure should however always be used with caution and only for solutions well buffered with *salts*.

In tables 4–9 will be found numerous comparisons which we have made between P_H values determined before and after dilution. In most cases they are in substantial agreement.

The dilution method if used with caution and understanding will, we believe, prove to be most useful to the bacteriologist. If for instance, one has to determine the P_H values of a hundred or so cultures and relative values which are approximately accurate are all that are necessary these relative and approximately accurate values may be obtained with remarkable rapidity by diluting 2 cc. of each of the cultures to 10 cc. with distilled water and measuring these diluted solutions. This leaves plenty of each culture for confirmatory tests or for other tests. Very highly colored or very turbid solutions may be diluted to a point where they may be used in the comparator.

Indicator papers may be mentioned in this section. A fair indicator paper may be made by impregnating paper with a mixed alcoholic solution of methyl red and brom thymol blue. We mention this particular combination because it may prove useful for roughly determining the reaction of solutions which vary widely in P_H. Such use is about the only one to which

indicator papers may safely be put. If the paper is not sized absorption phenomena seriously interfere. If the paper is sized the sizing is generally a buffer and destroys the sensitiveness of the test. The subject of indicator papers is worthy of more extensive investigations such as those of Walpole (1913), but at present it must be considered to be in an unsatisfactory state.

Often it is necessary to determine only the direction and the approximate extent of a fermentation. With the proper indicators this can be done much more rapidly and satisfactorily than by titration. If, for instance, the original medium was nearly neutral the addition of brom cresol purple will show in a second the approximate extent of an acid fermentation.

Again, in adjusting the reaction of culture media there are many instances where approximate adjustment is quite sufficient. With a series of indicators such as we have described one who is familiar with the colors at different P_H values can adjust media with a fair degree of accuracy by eye alone. At this point we may again call attention to the fact that adjustment of media to a given "percentage acidity" or "degree Fuller's scale" may result in greater divergencies in P_H than even an unskilled worker without the aid of comparison solutions attains when he adjusts by the colorimetric method.

In testing acid fermentations of a more or less homogeneous group of organisms it is often found that many of the cultures have arrived at about the same P_H. In this case it is convenient to test a few most carefully, and then arrange the other cultures in groups to match those tested. When such a procedure is permissible[16] the colorimetric method of testing acid fermentations becomes one of such rapidity that the burden of the tests is transferred from the analyst to the media maker and the inoculator.

[16] Methyl red cannot be left exposed to active cultures without danger of reduction or destruction.

SECTION XII. EQUIPMENT

The standard comparison solutions have been described in section V. The container for the N/5 NaOH solution should be paraffined. We have found the most satisfactory paraffined bottles to be those coated *thickly* with paraffine. We use about 1 pound of paraffine to a 5 litre bottle, cool it before it has time to form a crystal-like structure, and take particular pains to make sure that the bottom of the bottle is thickly covered. If the paraffine to be used is dirty it may be washed in hot distilled water, and the paraffine drawn off and dried at its melting point. The NaOH solution should be protected from CO_2 by efficient cotton-protected soda-lime tubes, and should not be brought in contact with rubber tubing but only with Jena or Pyrex glass tubing. This also may be paraffined. A convenient arrangement which requires no expert glass-blowing is shown diagrammatically in figure 6.

The containers for the other stock solutions may be of Jena or other resistant glass, and need not be protected from CO_2. A sample bottle is shown in figure 7.

The 50 cc. burette for the NaOH solution and the 50 cc. pipettes used in delivering the solutions which enter into the standard mixtures should be *calibrated* and kept *clean*. It is advisable to use only such volumetric apparatus as the Bureau of Standards has specified to be fit for test. [See Bureau of Standards Circular No. 9.] It will be noted that the Bureau will not accept for test the so-called "Shellbach" burettes.

As mentioned in section V, we find it convenient to prepare in a 200 cc. flask 200 cc. portions of each of the standard mixtures whose intervals are 0.2 P_H. This requires with the duplicates mentioned on p. 27 about forty-eight 200 cc. bottles each of which is provided with a 10 cc. pipette. These pipettes need *not* be calibrated and may be of rapid delivery. The bottles used for the alkaline borate mixtures should be paraffined. Two such bottles are shown in figure 7. Only a few bottles are needed for restricted researches.

The test tube racks, one of which is shown in the photograph,

are useful for many purposes but chiefly to hold the tubes of standard comparison mixtures. The tube holders are ordinary

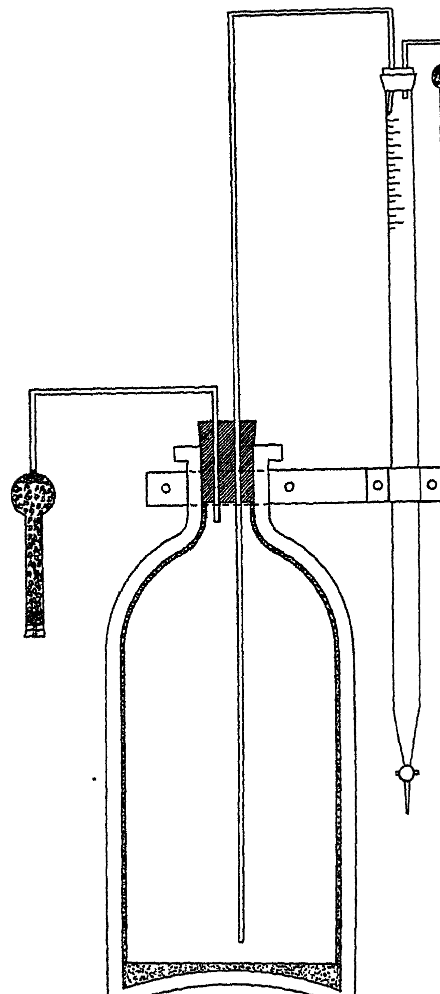


Fig. 6

metal rubber-stamp holders which may be purchased at any stationer's. We find that a row of nine accommodates all the tubes which it is advisable to use with one indicator, if the inter-

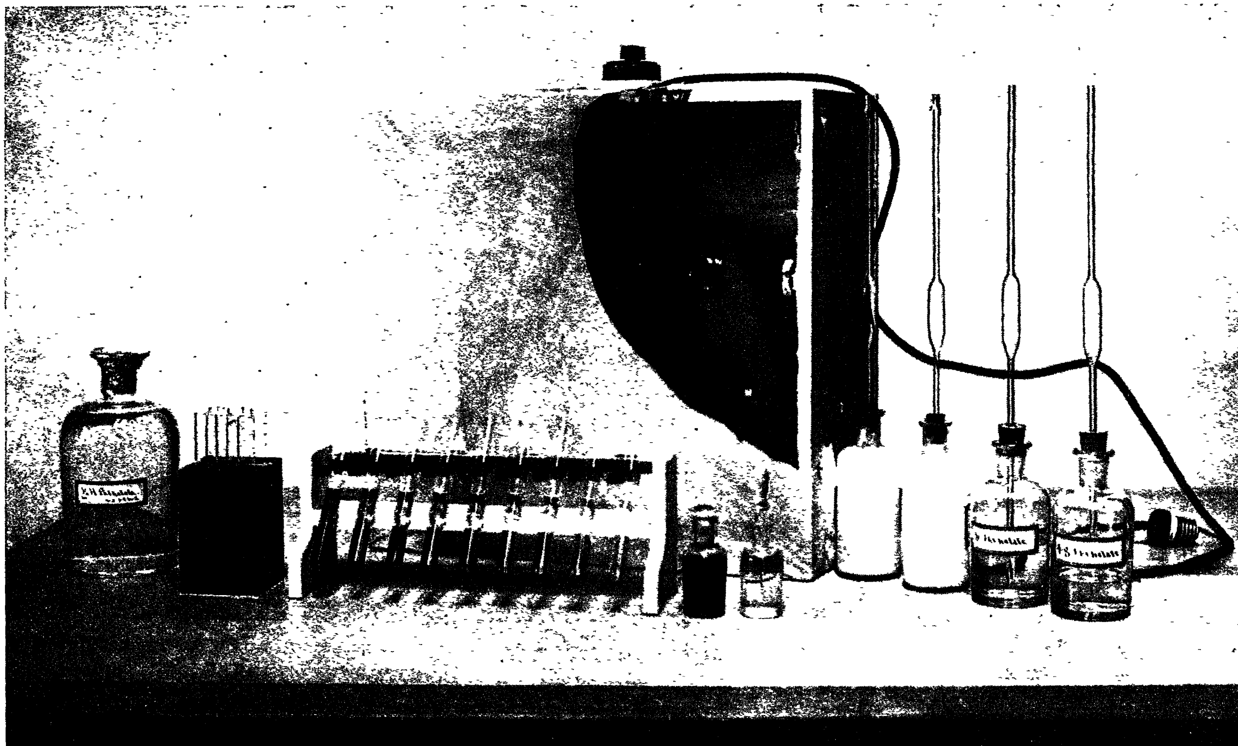


FIG. 7. Photograph showing screen, test tube rack, type of paraffined bottles used for alkaline standard mixtures, type of "acid resisting" bottle used for the acid standard mixtures, a Jena glass bottle such as is used for the acid standard stock solutions, two types of indicator droppers, and a "comparator."

vals are 0.2 P_H. A white background may be provided by using replaceable sheets of white paper.

The test tubes used should be selected for uniformity of bore and for clearness. It is hoped that an urgent demand will be created for a good grade of flat bottom tubes suitable for these tests.

The comparator of Hurwitz, Meyer and Ostenberg is shown in the photograph. We find $\frac{1}{2}$ inch holes instead of slits most suitable for a proper view. The interior as well as the exterior of the block should be painted a dull or "flat" black.

Two styles of indicator droppers are shown in the photograph. Neither will deliver accurately uniform drops, but either is satisfactory for ordinary purposes. For very careful work volumetric delivery of a dilute indicator solution may be used or a dropper made from a small burette with an orifice of capillary tubing with polished face. Such a burette should be mounted where it is as free as possible from tremors.

The screen for use with brom phenol blue and brom cresol purple is shown in the photograph with part of the screen torn away to show the arrangement of the lights behind. The device consists of an ordinary box of convenient size in which are mounted three or four large electric lights (e.g., 30 cp. carbon filaments.) A piece of tin serves as reflector. The box may be lined with asbestos board. A piece of glass cut to fit the box is held in place on one side by the asbestos lining and on the other by a few tacks. This glass serves only to protect the screen and is not essential. The screen is made from translucent paper known to draughtsmen as "Economy" tracing paper. It is stretched across the open side of the box and painted with a solution consisting of 5 cc. of 0.6 per cent phenol red (stock solution of phenol sulfon phthalein) and 5 cc. of $M/5$ KH_2PO_4 (stock, standard phosphate solution). While the paper is wet it is stretched and pinned to the box with thumb tacks. This arrangement may be constructed in a very short time and will be found very helpful in many cases. It should be used in a dark room or, if such a room is not available, exterior light may be shut off with a photographer's black cloth.

The indicator solutions which we have found convenient are the following. Phenol red [phenol sulfon phthalein] may be purchased as a standardized 0.6 per cent solution of its mono-sodium salt.[17] From this stock the solution used in the indicator tests may be prepared by diluting 10 cc. to 300 cc. in distilled water. This gives a 0.02 per cent solution. The other sulfon phthalein indicators may be purchased in solid form but it will be advantageous to have the manufacturer supply standardized stock solutions of the mono-sodium salts. In this case cresol-red should be furnished in the same concentration as phenol red, and the others in double this concentration, namely 1.2 per cent solutions. In all cases dilute 10 cc. of the stock solution to 300 cc. with distilled water to obtain the concentration used in the tests. Methyl red and propyl red solutions are prepared by dissolving 0.1 gram in 300 cc. alcohol and diluting to 500 cc. with distilled water. If solutions with strong buffer action are to be tested with methyl red it is permissible to use the following aqueous solution of this indicator. To a weighed amount of the finely ground indicator (0.5 gram) add slightly more than one molecular equivalent of NaOH (20 cc. M/10). Dilute to a 0.02 per cent solution for the tests. Ortho cresol phthalein (or the less brilliant phenol phthalein which may be used in the same range) is used in 0.02 per cent alcoholic solution.

The indicators required in the study of any particular range of P_H may be chosen from table 2, Section vi, Journal of Bacteriology, Vol. II, p. 33. This table includes the chemical names used in purchasing, the common names suggested for laboratory parlance, the concentration of the solutions used in the tests, the gross color changes, and the range in which each indicator is useful.

SECTION XIII. RÉSUMÉ OF GENERAL PROCEDURES

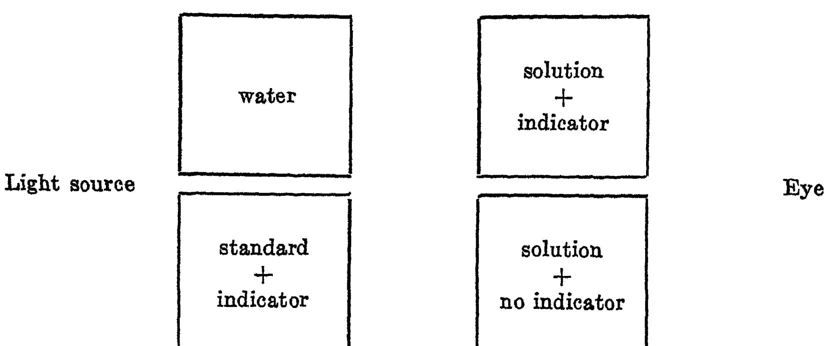
If the approximate P_H of the solution is not known, find the range within which it falls by adding to a portion either a mix-

[17] This solution is used by physicians for a renal function test and is supplied to them in small ampoules. The bulk solution specially made up for indicator purposes and free from carbonate, should be specified when ordered.

ture of methyl red and thymol blue, or else the sulfon phthalein series in succession beginning with thymol blue.

For clear, colorless solutions, use the standard comparison solutions (Section v) (10 cc. each). in test tubes held in a rack. Measure 10 cc. of the solution to be examined into a test tube of the same bore as that of the test tubes holding the standards. To it and to each of the standard solutions add the same number of drops of indicator solution. Four drops of the solutions we describe is generally sufficient, but judgment must be used. If, for example, phenol red is in question, it may be employed further in the alkaline region if used in lower concentration, and further in the acid region if used in higher concentration. In general however it is better to keep the indicator concentrations uniform and thus avoid confusion. The solution tested is now matched with the standards.

For colored and turbid solutions; if color and turbidity are slight, the solution may be treated as are the clear colorless solutions; as the color or turbidity increases use first the comparator or the dilution method and finally both. In the use of the comparator the tubes are arranged as follows.



Turbid solutions must be viewed through thin layers as from the side of a test tube. With the two indicators brom phenol blue and brom cresol purple the solution, if turbid, should be viewed in the screened light.

For special methods see other sections.

(*to be continued*)

SOIL FLORA STUDIES[1]

PART II

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III. SPORE-FORMING BACTERIA IN SOIL

Historical

No soil microörganisms have been studied more frequently or more completely than the group of spore-formers. They are constantly present in soil and are always found on gelatin or agar plates made from soil, producing the largest and most striking of the colonies that develop on such plates. They grow rapidly on ordinary media and, if a short period of incubation is used, they are among the most numerous kinds of bacteria developing on the plates. Literature relating to them has been concerned in part with their activities and in part with their taxonomy. From the point of view of the present paper, the literature relating to their taxonomy is the most important.

The importance of studying the botanical relationships of these bacteria was first emphasized by Meyer (as credited to Meyer by Gottheil, 1901), who laid great stress on the need of accurate measurements and careful descriptions. The first important contribution to the taxonomy of the group was by Gottheil (1901), one of Meyer's students. The same work was continued a few years later by Neide (1904), another of Meyer's students. Chester (1903) made a comparison of Gottheil's cultures with those obtained from other sources, and

[1] These papers together with those in the preceding number of the Journal are original abstracts of Technical Bulletins Nos. 57 to 60 of the New York Agricultural Experiment Station.

somewhat simplified Gottheil's classification. Chester's work and Neide's work were both in progress at the same time, without either investigator having access to the other's cultures; so further work was necessary in order to harmonize their findings.

This coördination has been accomplished by the recent work of Ford and his associates (1916). They obtained a larger number of cultures than had been studied by any previous investigators, some of their own isolation, some from Kral's collection, some from the American Museum of Natural History, and some from other sources. With such a large number of cultures, it was possible to study correlations and observe differences to better advantage than had been previously possible, and their findings have a correspondingly greater authority.

The three most common species

Three types of spore-forming bacteria have been found in all the soils studied and have always been observed on the plates, unless too great dilutions have been used. They have been satisfactorily identified with: *B. megatherium* DeBary, *B. mycoides* Flügge, and *B. cereus* Frankland.[2] In each case the identification has been regarded as correct, because the cultures isolated from soils agree well with the published descriptions given by Chester and by Ford *et al.*, and also because when representative cultures were sent to Ford for identification, he assigned to them the same names as those already decided upon by the writer.

Of these three species, *B. megatherium* has proved to be slightly more abundant than the other two, plate counts averaging about 375,000 per gram. Plate counts of *B. mycoides* have averaged about 225,000 per gram, and of *B. cereus* about 180,000. These findings are similar to those recorded by Ford and his associates, the chief difference being that they found *B. mycoides* comparatively rare in soil and found *B. petasites* Gottheil more numerous than either *B. mycoides* or *B. megatherium.*

[2] Descriptions of these three species are given in Technical Bulletin No. 58 of the New York Agricultural Experiment Station.

Less common species

Besides the three species present in all of the soils studied, a number of other types have been isolated. Only one of these, *B. simplex* Gottheil, has been found often enough to justify detailed study.[3] It is not possible to say whether *B. simplex* occurs in all of the soils studied, because it does not have a distinctive colony like the three more common species and does not occur in sufficient abundance to be encountered regularly in a series of chance isolations. In making chance isolations, *B. simplex* has been found about a fifth as often as *B. megatherium*, *B. mycoides*, or *B. cereus*. The identification of these cultures as *B. simplex* is considered to be correct, as they agree with Chester's and with Ford's descriptions of the species, and the identification has been confirmed by Ford in the case of a typical culture sent to him.

Among the other types of spore-forming bacteria encountered were a few cultures of small-spored organisms closely related to *B. mesentericus* (Flügge) Migula, two cultures of an organism with round, terminal spores, probably *B. fusiformis* Gottheil, and several others that have proved difficult to identify.

Gelatin colonies of the spore-forming bacteria

In making a flora study of soil, it is quite important to know the relative abundance of the different types of organisms studied. About the only way to establish this is by means of the relative numbers of the colonies of these organisms which appear on plates made from soil; and in order to count the colonies of any type, it is necessary that they be readily distinguishable from the colonies of other bacteria. On gelatin, the three most common spore-formers of soil produce colonies that can be recognized fairly readily.

Unfortunately it is not possible to describe the colonies of these bacteria so that they can be recognized with certainty by other investigators, because of differences in form of colony

[3] A description of this species is given in Technical Bulletin No. 58 of the New York Agricultural Experiment Station.

due to differences in technic. In the present work, *B. mycoides* has been recognized by its large, rapidly liquefying, filamentous to rhizoid colony; *B. cereus* by its fair-sized, round, rapidly liquefying colony with entire edges and a surface membrane containing granules that are generally concentrically arranged; *B. megatherium* by its smaller colony, containing a flocculent center composed of white opaque granules, generally surrounded by a zone of clear liquefied gelatin. Using these criteria, it has proved possible to obtain a fairly good estimate of the numbers of each of these three species in the different samples of soil investigated.

Significance of the spore-forming bacteria in soil

The great attention that has been given in the past to the spore-forming bacteria of soil is undoubtedly due to their constant presence in soil, their large striking colonies on agar or gelatin plates, and their rapid growth in laboratory media. Chester (1902), for example, used fairly short periods of incubation, and because of the rapid growth of these organisms, found them to comprise nearly half of the colonies on his plates. Hiltner and Störmer (1903) on the other hand, incubated their plates until no more new colonies appeared, and found only 5 per cent of the colonies to be liquefiers (which category includes nearly all, if not all, of the spore-forming bacteria). The use of short incubations has led to an exaggerated idea of the abundance and importance of spore-forming bacteria in soil. It was learned that they were vigorous ammonifiers in laboratory culture media, and naturally it was assumed that they were also vigorous ammonifiers in soil. This idea has persisted in spite of recent observations as to their relatively small abundance.

It was recently pointed out by the writer (1916 b) that the number of colonies of the spore-formers developing on plates per gram of soil was not appreciably diminished by heating the soil infusion, before plating, to a temperature high enough to kill vegetative forms. The results indicate that these bacteria exist in normal soil only in the form of spores and are

therefore ordinarily inactive. Since writing the paper just mentioned, further information on the subject has been obtained by means of the microscopic examination of soil; and it has entirely corroborated this conclusion.

Ford and his associates (1916) have found practically the same kinds of spore-forming bacteria not only in milk, in water, and in soil, but also in dust, and always in about the same relative abundance. This probably means that the spores of these bacteria are omnipresent, but that they become active only when conditions are favorable. It is not impossible that the reason why these species have developed the power of producing spores is because the conditions necessary for their growth are so rarely met in nature that their continued existence would be impossible without some resting stage. Probably in soil, conditions favorable to their growth occur just often enough so that the spores do not diminish in numbers; but at other times they are dormant.

IV. NON-SPORE-FORMING BACTERIA IN SOIL[4]

Introduction

The non-spore-forming bacteria seem to be the most abundant group of microörganisms in soil; but much less is known about them than about the spore-formers. It has so far proved impossible to distinguish the different kinds of non-spore-formers from each other, or to obtain definite knowledge as to their function in soil. This paper therefore is merely preliminary. Its object is primarily to call attention to the probable importance of non-spore-forming bacteria in soil.

Spore-forming bacteria and Actinomycetes have been extensively studied in the past; but except for the nitrifiers and some of the other organisms concerned in the transformations of nitrogen, scant attention has been given to any non-spore-forming bacteria. As an illustration of this fact, mention may be made of Löhnis' review of the subject in his "Handbuch der

[4] This section of the paper was presented at the New Haven meeting of the Society of American Bacteriologists.

landwirtschaftlichen Bakteriologie." In his section (p. 514) entitled "Allgemeines über die im Boden vorkommenden Arten von Mikroorganismen" he refers to non-spore-forming bacteria in just two sentences. In these two sentences he says that except for the fluorescent varieties, attention has been given only to certain yellow and blue chromogenic forms. In other words, the large group of non-chromogenic bacteria that produce punctiform colonies on agar or gelatin plates has been practically overlooked.

Considering what a large proportion of the colonies on the plates are of this type, it seems strange at first thought that they have been overlooked; and yet the reason is not far to seek. In laboratory culture they grow poorly and their activities are so insignificant that they can scarcely be measured by chemical tests. It has been assumed that organisms so inactive in the laboratory must also be inactive in soil.

Classification

Two main groups of non-spore-formers have been recognized in this work. The first is the group that liquefies gelatin rapidly, the second the group that produces mere punctiform colonies in gelatin. The latter group contains some slow liquefiers, and some non-liquefiers. At first thought it would seem as though a much more natural division to make would be to distinguish liquefiers from non-liquefiers, but that has not proved to be the case. The bacteria found in the soil which liquefy gelatin slowly seem to be more nearly related to those which do not liquefy it at all than to those which liquefy rapidly.

This division has also proved easier to recognize in practice than it would have been to draw the line between liquefiers and non-liquefiers. Many slow-liquefiers do not begin to liquefy at the end of seven days' incubation, and cannot be distinguished from non-liquefiers by means of their colonies. The rapid-liquefying group, however, can always be distinguished by the fact that its colonies are over 1 cm. in diameter, and if given time often liquefy the entire plate; while the colonies of the other

group never become as large as 1 cm. in diameter, however long the incubation.

The most important member of the rapid-liquefying group is *Ps. fluorescens* (Flügge) Migula. For convenience' sake, therefore, the group may be spoken of as the *Ps. fluorescens* group; but this expression must not be taken to mean that all the organisms are fluorescent. Many of them, indeed, never produce fluorescence, and non-fluorescent strains of *Ps. fluorescens* itself are often encountered. The rapid liquefaction of gelatin is the most striking common characteristic of this group. No non-liquefying fluorescent organisms have been encountered. Besides *Ps. fluorescens*, a number of other members of the rapidly liquefying group have been found. They are small, short rods, generally with polar flagella, and seem to be closely related to *Ps. fluorescens*. They are generally present in soil, although not in large numbers. None of them have been identified with previously described species. Only one of them has definite enough characteristics to be easily recognized. It is a very slender rod (about 0.2 to 0.3 micron in diameter) that produces a characteristic orange-colored colony in gelatin. No study has yet been made of any of the other forms.

The other group of asporogenous bacteria here discussed, probably does not contain any well-known organism. It is a group of small, short rods or cocci, rarely over 1 micron in length or 0.5 micron in diameter, characterized by poor growth in all liquid media, and by the production of punctiform colonies in gelatin. On agar streak culture they grow fairly well, producing a soft, smooth, glistening, slimy to watery growth. In several of the writer's publications they have been spoken of as the group of "slow-growers." This term, however, gives a false conception of their importance in soil; for in soil, conditions probably favor their growth, and there is reason to believe that they multiply rapidly there.

The actual number of different kinds of bacteria in this group is unknown. There are very few morphological differences upon which to base distinctions; and because of their poor growth in liquid media, it is not possible to use the ordinary

physiological tests to effect a classification. Some new plan of classification must be applied to these organisms, but no satisfactory system has yet suggested itself. The following classification is all that has been attempted in the present work.

- I. Rod-shaped forms.
  - A. Chromogenic (yellow).
  - B. Non-chromogenic.
    - 1. Liquefying gelatin slowly.
    - 2. Not liquefying gelatin.
- II. Cocci.
  - A. Liquefying gelatin slowly.
  - B. Not liquefying gelatin.

Of these different groups, the cocci are much less abundant than the rod-shaped forms. Most abundant of all are the non-chromogenic rods that liquefy gelatin slowly.

Recognition of colonies on plates

Unfortunately the non-spore-forming bacteria in general produce colonies on gelatin and agar that do not have striking characteristics. *Ps. fluorescens* and the orange liquefying type are the only exceptions to this statement. *Ps. fluorescens* can be recognized on gelatin by its large, rapidly liquefying colony, with entire margin, faintly cloudy but almost structureless; but its agar colonies are less characteristic, fluorescence generally occurring on beef-extract-peptone agar, but not constantly enough to characterize the species. The orange, liquefying type can be recognized by its fairly large orange colonies; on gelatin they are sometimes as large as 2 cm. in diameter, and show a striking radiate structure.

The other non-spore-forming bacteria all produce non-characteristic colonies. The punctiform colonies can be distinguished from the rapidly liquefying ones; but that is all the classification that can be made. This is unfortunate, because it is impossible to tell how numerous any type is in a particular soil sample unless its colonies on the plates can be recognized. Before a satisfactory study can be made of this part of the soil flora, a new medium must be devised upon which the colonies of the asporogenous types produce characteristic colonies.

Distribution in soil

The non-spore-formers have been found in general to average about 75 per cent of all the flora of cultivated soil developing on gelatin plates. In sod soil—in which, as already stated (Conn, 1916 a,) the number of Actinomycetes is higher than in cultivated soil—the percentage of non-spore-formers is somewhat lower. The majority of this 75 per cent are "slow-growers." The rapid-liquefying group is not a very abundant one in the soils studied. In many cases it is either lacking or not abundant enough to appear on plates in the dilutions used; and the largest number ever found, except in manured soil, is about 1,500,000 per gram of soil or about 5 per cent of all the colonies developing on the plates. The group of "slow-growers," on the other hand, is very abundant. Excluding air-dried soil, where their numbers are quite low, and manured soil, where their numbers are extremely high, plate counts have ranged from about 2,000,000 per gram to about 60,000,000 per gram.

The mere fact that the majority of the bacteria developing on plates made from soil are non-spore-formers suggests that these bacteria are important in soil; but still stronger evidence of their importance comes from the fact that they fluctuate greatly in numbers. In this respect they differ greatly from the spore-forming bacteria, which as already mentioned (Conn 1916 b) are relatively constant in numbers. In one series of samples of field soil taken from one plat at intervals for three years, the plate count of non-spore-formers has varied from 5,000,000 to 44,000,000 per gram; while all the other organisms growing on the plates (spore-forming bacteria and Actinomycetes) have varied only from 3,200,000 to 10,500,000 per gram. In another series of tests of field soil from a different locality, carried on throughout a period of two years, the number of punctiform colonies (complete counts of the non-spore-formers not having been made in this case) varied from 2,000,000 to 25,000,000 per gram; while the number of all the other colonies varied only from 1,700,000 to 8,800,000 per gram.

Even more striking fluctuations have been observed in pot

experiments. The aeration brought about by potting soil is generally admitted to cause an increase in the numbers of bacteria. In the course of the present work it has been noticed that the numbers of spore-formers and Actinomycetes remain about constant after aeration, and that only the non-spore-formers increase noticeably. A good illustration of this is given in table 1, in which it can be seen that during the first week or two after aeration the count of rapid-liquefying non-spore-formers rose to about four times its normal height, the count of punctiform colonies to twice normal, while the number of colonies of all other kinds remained constant.

Table 2 shows an even more striking illustration of the fluctuations in numbers of non-spore-formers. In this case the effects of manuring are added to the effects of aeration. During the two weeks after manuring the number of rapid-liquefying colonies of non-spore-formers and the number of punctiform colonies have each increased to about one hundred times normal, while the number of colonies of all other kinds have not more than doubled (which apparent doubling in number may be due to the inaccuracy introduced by the high dilutions necessary).

TABLE 1

Plate counts of aerated soil

TIME SINCE AERATION	NON-SPORE-FORMING BACTERIA				OTHER COLONIES (SPORE-FORMERS AND ACTINOMYCETES)
	Rapid-liquefying colonies		Punctiform colonies		
	Per gram	Per cent	Per gram	Per cent	Per gram
(Unaerated)	350,000	2.2	11,000,000	73.5	4,700,000
1 day	1,500,000	5.5	22,500,000	80.5	4,000,000
5 days	1,250,000	5.5	18,000,000	79.0	3,000,000
13 days	1,500,000	5.0	24,000,000	78.5	4,500,000
22 days	1,000,000	5.0	16,000,000	79.0	5,000,000
3 months	300,000	2.2	10,000,000	77.0	3,200,000
3 months, 25 days	700,000	2.2	27,500,000	85.5	4,500,000
4 months, 8 days	300,000	1.6	16,000,000	85.0	2,700,000
4 months, 10 days	600,000	2.3	23,000,000	85.0	2,400,000
4 months, 15 days	350,000	1.5	19,000,000	80.0	4,700,000
7 months, 15 days	150,000	1.0	12,000,000	76.0	4,000,000
10 months	100,000	1.2	4,800,000	60.0	3,000,000

TABLE 2

Plate counts of manured soil

TIME SINCE MANURING	NON-SPORE-FORMING BACTERIA				OTHER COLONIES (SPORE-FORMERS AND ACTINOMYCETES)
	Rapid-liquefying colonies		Punctiform colonies		
	Per gram	Per cent	Per gram	Per cent	Per gram
(Unmanured)	100,000	1.0	3,400,000	34.0	6,500,000
1 day	4,700,000	2.3	190,000,000	95.0	5,000,000
3 days	25,000,000	6.0	370,000,000	93.0	5,000,000
6 days	28,000,000	8.8	280,000,000	90.0	12,000,000
9 days	29,000,000	18.0	125,000,000	78.0	6,000,000
15 days	38,000,000	19.0	150,000,000	77.0	12,000,000
19 days	10,000,000	15.0	48,000,000	73.0	8,000,000
1 month	4,500,000	6.5	53,000,000	76.0	12,500,000
1 month, 15 days	2,000,000	4.5	32,000,000	72.0	10,000,000
2 months	2,300,000	6.5	27,000,000	73.0	6,700,000
4 months	1,500,000	6.8	15,000,000	67.5	5,500,000
6 months	150,000	1.3	7,000,000	66.0	3,850,000
8 months	450,000	3.0	8,500,000	55.0	6,500,000
12 months	200,000	2.5	4,200,000	52.5	3,600,000

Significance of the non-spore-forming bacteria in soil

The great abundance of non-spore-formers in soil is not necessarily proof that they are important. It has been emphasized again and again in the past that the most abundant bacteria in soil may not be the most important ones. It has been insisted that the function of any kind of organism must be shown before its importance in soil can be admitted. This argument is unquestionably correct, but has caused too much emphasis to be laid upon the functions of the organisms rather than upon their abundance. It has often been assumed that because a given type of soil microörganism can produce a certain chemical change in pure culture, it is the cause of the same chemical change in soil, without further investigation to see whether it occurs in active form in soil in which that chemical change is taking place, or whether the active individuals are numerically important. This error was made when it was assumed that members of the *B. subtilis* group were important ammonifiers in soil.

The conclusions drawn in the present work do not depend upon mere numerical predominance of the non-spore-forming bacteria. They are based on the fact that when any change in soil conditions causes an increase in the number of bacteria present, the greatest increase always seems to occur in the group of non-spore-forming bacteria. This plainly indicates that they are active in soil; and considering that they comprise over half the soil flora, it is hard not to believe that they are important.

From the small amount of information at hand, however, it is impossible as yet to draw definite conclusions as to their function. *Ps. fluorescens* is known to be an ammonifier, and in the past has been acknowledged to be abundant in manure. This organism, together with the other rapid-liquefying types, have unquestionable proteolytic powers, and are probably active in the decomposition of manure (and perhaps other organic matter). The "slow-growers," however, liquefy gelatin so slowly (if at all) that the extent of their proteolytic ability is questionable, and their activity in soil remains an unsolved problem. Nevertheless the increase in punctiform colonies after the addition of manure to soil suggests that in this experiment at least they took part in some phase of the decomposition of manure.

The nitrifying organisms originally described by Winogradsky are asporogenous bacteria, and the idea has suggested itself that nitrification might be the function of the organisms studied in the present work. All the present cultures, however, were obtained from gelatin plates and it is not likely that any of the forms which grow on gelatin are nitrifiers, since Winogradsky's nitrifiers did not grow on gelatin. Nevertheless the possibility is not entirely ruled out of court, because in the present work the gelatin has been of a different composition and the general technic quite different from that used by Winogradsky and others who have studied the nitrifiers. For this reason about ten cultures, isolated from punctiform colonies, were inoculated into a mixture of sand and Omeliansky's solution, but no signs of nitrite appeared. No definite statement, of course, can be made on the basis of such meager data, and when a more com-

plete study of these organisms is made, nitrifying power is one of the tests that must be included. For the present their function in the soil remains a matter of speculation.

V. ACTINOMYCETES IN SOIL

The third large group of soil microörganisms, the Actinomycetes, are generally spoken of as higher bacteria, but are sometimes considered to belong with the higher fungi rather than with the bacteria. Especial attention is now being given to these organisms at the New York Agricultural Experiment Station. A study is being made of their physiological activities in soil and in culture media, and of the constancy of the various characteristics that may be used for the separation of species. The present paper is merely a report of progress on this study. A more complete publication on the subject may be expected later.

Historical

The question of nomenclature. The first species described well enough so that it can possibly be recognized today is *Actinomyces bovis* Harz 1877, the cause of bovine actinomycosis. The name *Actinomyces*, however, is by no means generally accepted for this genus. The names *Streptothrix, Nocardia, Cladothrix* and *Oospora* are often used to refer to this genus or to some part of it. Of these names *Streptothrix* is used nearly as often as *Actinomyces*, but is untenable, because it was preëmpted by Corda in 1839 for a genus of true fungi. *Cladothrix* is applied to a definite genus of higher bacteria (of which *C. dichotoma* Cohn is the type) which is distinctly different from *Actinomyces*. *Oospora* is the name of an ill-defined genus of higher fungi, of which the best known member, *O. lactis*, bears no resemblance to *Actinomyces*.

The only tenable generic names for these organisms are *Actinomyces* Harz, 1877 and *Nocardia* Trevizan, 1889. Of them, *Actinomyces* must be preferred on grounds of priority, provided only one generic name is used for all these organisms. *Nocardia* may be used for one section of the group when it is subdivided;

but attempts to subdivide it have so far proved unsuccessful. Eventually it will probably be broken up; but the recognition of genera must be preceded by the recognition of species, and as yet there is no species definitely enough described to serve as a type for *Nocardia.* Besides animal pathogens, perhaps the only recognizable species is the cause of potato scab, which was originally called *Oospora scabies* by Thaxter. For the present the genus *Actinomyces* Harz, em. Gasperini, is best left undivided.

Classification. The literature is full of names of species of Actinomycetes that have not been described sufficiently for recognition. Early investigators cultivated these organisms on the ordinary complex organic media which are of variable composition and are not especially adapted to the growth of these peculiar types. On such media their growth is likely to be variable and non-characteristic; which fact prevents the recognition today of most of the species described in earlier years.

Krainsky (1914) was the first to describe the characteristics of these organisms on media of definite chemical composition. His work was important in calling attention to the value of such media; but even his species are difficult to recognize. Waksman and Curtis (1916) also used media of definite chemical composition; but as they did not use Krainsky's media, it is impossible to tell whether their species are new or are the same as previously described by Krainsky. It has scarcely been recognized in the past what remarkable differences the same species may show when growing on media of slightly different composition.

Methods of characterization

In the present work, most stress has been laid upon cultural features in characterizing types. There is reason to hope, however, that in a final classification the broadest sub-divisions may be based upon differences in morphology. There are some quite striking differences in shape and size of conidia and filaments; but a thorough study of their constancy must be made before they can be used for the purpose of classification.

The greatest aid yet found in classifying these organisms has

been the use of glycerin in synthetic media. The use of glycerin agar for stimulating their growth is not a new idea. It was used in the earliest studies on Actinomycetes; but apparently the early investigators added glycerin to the ordinary complex organic bacteriological media and so failed to obtain its full value in the separation of species. The best media so far found for this purpose are agar media containing 1 per cent of glycerin and either malic or citric acid. On these media the growth may be of almost any color in the spectrum, and almost as many different colors have been observed in the aerial mycelium as in the mass of growth beneath; while the medium itself may be colored red, orange, brown, gray, yellow, green or blue. The colors observed in the medium, in the growth, and in the aerial mycelium frequently differ greatly from each other; so the number of possible variations in chromogenesis is almost endless. The pigment production by any one strain in the same medium, however, is fairly constant.

Other agar media of definite chemical composition have also proved of some use. The test for nitrate reduction is also of value in characterizing types; but special media must be used, because of the poor growth of Actinomycetes in the ordinary nitrate broth.

The results of this investigation of methods have shown the fallacy of trying to establish species at present. Every new medium that has been devised has served to break up still further the types already recognized. This process is likely to continue for some time, as new media are investigated. The only way in which previously described species can be recognized after new methods are used in classification is to study cultures of the original strains obtained from their authors.

Classification

About three hundred cultures have been studied. They have been classified into about seventy different types by means of the tests just mentioned. As the cultures were obtained by the direct plating technic without preliminary cultivation or

incubation at special temperatures, and as no wide search for different varieties was made, the number of types present in soil may be twice or three times the number found. It is not impossible that the Actinomycetes are as rich in species and as diverse in function as the lower bacteria. Only three of the types recognized have been found to be of common occurrence in the soils investigated. None of them are to be considered distinct species.[5]

Only one of the types studied so far has been found to agree with any species previously given a definite description. Certain cultures have proved to have the characteristics of *A. diastato-chromogenus* Krainsky. These cultures, however, include strains of the potato scab organism, and if all one species, must be called *A. scabies*. This is one of the fairly common soil types; but as the pathogenicity of the cultures has not yet been tested, it is not known whether the potato scab organism has actually been isolated from the soils studied.

Recognition of colonies on plates

The best medium so far found for distinguishing the different types of Actinomycetes on plates is the asparaginate-glycerin agar mentioned in the second paper of this series. Unfortunately, however, some of the most common soil types produce non-characteristic colonies on it. Work is now in progress with the object of obtaining a more satisfactory medium for this purpose.

Distribution of Actinomycetes

The members of this group are very widely distributed in nature. As pathogens they do not seem to be as abundant as the lower bacteria; but as saprophytes their distribution compares with that of the true bacteria. Soil seems to be the natural habitat of the saprophytic forms. To anyone familiar with these organisms, the very odor of soil is proof enough of their abundance in it.

[5] Brief descriptions of the most abundant types are given in Technical Bulletin No. 60 of the New York Agricultural Experiment Station.

Their great abundance in soil was discussed in a recent publication (Conn, 1916 a) in which it was also mentioned that more Actinomyces colonies developed on plates from sod soil than on plates from cultivated soil. They were found to comprise about 20 per cent of the total flora in cultivated soil and about 37.5 per cent in sod soil. This difference between sod and cultivated soil in Actinomyces content is usually indicated even by the odor of the soil.

It has been noticed in the past that Actinomyces cultures can be isolated from the roots of certain plants. This, together with the observation just noted and with the fact that potato scab is caused by an Actinomyces, raised the question whether the presence of grass roots might not cause an increase in numbers of Actinomycetes in soil. The experiment has therefore been tried of mixing grass roots with soil and observing the effect on the Actinomyces content of the soil. A greater proportion of the colonies on the plates made from this soil were found to be Actinomyces colonies than on the plates made from similar soil without grass roots; nevertheless the results of the experiment can hardly be considered conclusive.

Significance of Actinomycetes in soil

The common occurrence of Actinomycetes in soil has led to many speculations as to their significance. Ammonification, nitrate-reduction, and cellulose-decomposition have at one time or another been definitely assigned to these organisms as their function in soil. Generally, however, such conclusions have been drawn from observations in pure culture, which do not necessarily show what their activities may be under soil conditions. It is very likely that certain types of Actinomycetes may carry on the activities assigned to them; but only a comparatively few varieties have been studied, and observations as to these few cannot logically be applied to the very diverse Actinomyces flora of soil.

The statement has sometimes been made that Actinomycetes are concerned in the decomposition of manure. In this respect,

the results of the experiment reported in the preceding paper (table 2) are rather surprising; for in that case the numbers of Actinomycetes developing on the plates remained almost constant after the addition of manure. Although this experiment by no means disproves their activity in the decomposition of manure, it emphasizes the fact that more work is needed before their part in such activities can be definitely determined.

As already mentioned (Conn 1916 a), the abundance of these organisms in sod soil suggests that they take part in the decomposition of grass roots. Whether this is the true explanation or not, the facts in regard to sod soil and soil to which grass roots are added indicate that Actinomycetes are active in soil. In this respect they are to be compared with the non-spore-forming rather than with the spore-forming bacteria.

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STUDIES IN THE NOMENCLATURE AND CLASSIFICATION OF THE BACTERIA

II. THE PRIMARY SUBDIVISIONS OF THE SCHIZOMYCETES

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Since the definite recognition of the fact that bacteria, for the most part at least, are to be classed with plants rather than with animals, several names have been proposed to designate the entire group. The following may be noted:

Schizomycetes Naegeli 1857, p. 760.
Bacteriaceae Cohn 1872 *a*, p. 237.
Bacteria Cohn 1872*b*, p. 136.
Schizomycetaceae DeToni and Trevisan 1889, p. 923.

The bacteria as a group have received different ranking from various workers. Their exact position in the botanical scheme has also received different interpretations. Some authors have regarded the bacteria as constituting a family, others have called the group an order, still others a class. The most commonly accepted ranking at the present seems to be to place the *Schizophyceae* (blue green algae) and the *Schizomycetes* as coördinate classes of the phylum *Schizophyta.* The name *Schizomycetes* apparently has priority as a class name, and is entirely suitable and valid. It may be characterized as follows:

Schizomycetes Naegeli (1857, p. 760) char. emend. Migula (1894, p. 235)

Typically unicellular plants, cells usually small and relatively primitive in organization. The cells are of many shapes, spherical, cylindrical, spiral or filamentous; cells often united into groups, families or filaments; occasionally in the latter showing some dif-

ferentiation among the cells, simulating the organization seen in some of the blue-green, filamentous algae. No sexual reproduction known. Multiplication typically by cell fission. Endospores are formed by some species of the Eubacteriales (see below), gonidia (conidia, arthrospores) by some of the filamentous forms. Chlorophyll is produced by none of the bacteria (with the possible exception of a single genus). Many forms produce pigments of other types. The cells may be motile by means of flagella; some of the forms intergrading with the protozoa are flexuous, a few filamentous forms (as Beggiatoa) show an oscillating movement similar to that of certain of the blue green algae (as Oscillatoria).

The bacteria have been subdivided in many different ways. Cohn (1872) recognized four main divisions, or families, as he termed them; *Spherobacteria* for the cocci, *Microbacteria* for the short, non-filamentous rods, *Desmobacteria* for the longer, filamentous rods and *Spirobacteria* for the spiral forms. Later, in his classification of 1875, the bacterial genera were distributed under the two tribes into which he divided the *Schizophyta* (comprising both the blue green algae and the bacteria). The tribe *Gloeogenae* included those forms in which the cells are not united into filaments and the tribe *Nematogenae* those in which the cells occur in filaments.

Winter (1879) did not recognize groups among the bacteria higher than the genus, except that he placed the genera *Sphaerotilus* and *Crenothrix* in an "Anhang" to the bacteria.

Zopf (1883) subdivided the bacteria into four families, *Coccaceen, Bacteriaceen, Leptothricheen,* and *Cladothricheen.* The first family included only *Leuconostoc,* a spherical organism occurring in chains, the remaining families all included cocci, short rods, long rods and filaments and, in the last family named, spirals, as stages in the growth of the organism. The second showed no differentiation of base and apex of filament, the third showed a contrast between base and tip and the fourth showed pseudobranching.

De Bary (1884) used as a primary division the ability of the organisms of one group to develop endospores and of the other to produce arthrospores.

Zopf (1885) expanded his classification of bacteria, but used the same four main divisions as in his earlier discussion (1883).

Hueppe (1886) followed De Bary in dividing the bacteria into two groups, those forming endospores and those with arthrospores. The family *Leptotricheen* is given as an Anhang. Later (1891) he abandoned the scheme here outlined as a primary grouping.

Flügge (1886) divided bacteria into those which are spherical, rod shaped, spiral and those with variable growth forms.

The work of Winogradsky (1888) on the sulphur bacteria emphasized their importance as a group perhaps coördinate with the true bacteria.

Schroeter (1886) divided the class *Schizomycetes* into three orders: *Cocobacteria* for the spherical forms, *Eubacteria* for the rod forms, and *Desmobacteria* for the larger filaments, usually possessing a definite sheath.

De Toni and Trevisan (1889) divided the family *Schizomycetaceae* into three subfamilies (equivalent to the orders of Schroeter). These are: *Trichogenae*, the filamentous, frequently sheathed bacteria; *Baculogenae* with isolated rod shaped cells or with cells in chains (not filaments); and *Coccogenae* with spherical cells.

Ludwig (1892) recognized as the primary divisions cocci, rods and filaments (usually sheathed).

Thaxter (1892) called attention to the existence of a very distinct group of bacteria which he included in his family, *Myxobacteriaceae*. These organisms are characterized by the development of a pseudoplasmodial motile stage and a fruiting stage in which complex fruiting structures resembling those of the slime molds are formed.

Hueppe (1895) used five coördinate divisions (families) *Coccaceae*, *Bacteriaceae*, *Spirobacteriaceae*, *Leptothricheae* and *Cladothricheae*.

Migula (1894, 1895, 1897) also recognized five families of bacteria, *Coccaceae*, *Bacteriaceae*, *Spirillaceae*, *Chlamydobacteriaceae* and *Beggiatoaceae*.

Fischer (1895) divided the bacteria into the two subclasses

Haplobacteriaceï and *Trichobacteriaceï*. The latter included those types whose vegetative phase consists of unbranched or branched filaments or chains of cells, the individual members of which break off as swarm spores or gonidia, the former those whose cells are not typically filamentous.

Lehmann and Neumann (1896) divided the microörganisms included by most authors among the bacteria into three coördinate groups, *Schizomycetes* or fission fungi (true bacteria), *Hyphomycetes* (later *Actinomycetes*) or thread organisms showing true branching and the *fission algae* including other filamentous forms evidently closely related to the algae.

Chester (1897) recognized six orders (families) of bacteria, *Coccaceae, Bacteriaceae, Spirillaceae, Mycobacteriaceae, Chlamydobacteriaceae,* and *Beggiatoaceae.*

Jennings (1899) proposed that the two principal groups of the bacteria should be *Paraschizae* and *Diaschizae,* to include organisms which multiply by longitudinal and by transverse division respectively.

Migula (1900) divided the class *Schizomycetes* into two orders, the *Eubacteria* or true bacteria and the *Thiobacteria* or sulphur bacteria.

Kendall (1902) used in the main the classification proposed by Chester, but merged the family *Mycobacteriaceae* with *Bacteriaceae.*

Fischer (1903) recognized the two orders *Haplobacterinae* and *Trichobacterinae.*

Erwin F. Smith (1905) followed Migula's (1900) scheme of ordinal designation, but added a third order, *Myxobacteria.*

The discovery by Schaudinn (1905) of the causal organism of syphilis and the interest during the past decade in the relapsing fevers and the protozoan diseases has called particular attention to the spiral microörganisms, particularly the flexuous forms usually termed spirochetes. There is no agreement at the present time as to the position of these forms either among the true bacteria or the protozoa. Blanchard (1906) put the genus *Spirochaeta* with *Trypanosoma* in the *Trypanosomidae.* Swellengrebel (1907) included these forms with the bacteria in the

Spirillaceae. Doflein (1911) placed them among the protozoa. Dobell (1911) used the group name *Spirochaetoidea*. Gross (1912) included them under *Spironemaceae*, as did also Gonder (1914).

Lotsy (1907) followed the bacterial grouping proposed by Fischer, but added *Myxobacteria* as a coördinate order.

The work of Ellis (1907) and of Molisch (1910) has emphasized the sheathed filamentous or iron bacteria and their importance as a bacterial group.

Jensen (1909) has proposed that the bacteria be divided into the two orders *Cephalotrichinae* and *Peritrichinae*, the former to include those bacteria which are the more primitive and are typically water forms; endospores produced only in a few sulphur free bacteria; cells spherical, rod shaped or spiral; securing their growth energy almost exclusively by oxidative processes; cells motile or non motile, if the former the flagella polar, never diffuse. In the *Peritrichinae* on the other hand, the cells are either spherical or rod shaped, never spiral; peritrichous or non-motile; not typically primitive water forms and usually not securing growth energy solely by oxidative processes.

Heim (1911) differentiates between the true bacteria or *Schizomycetes* and the thread bacteria or *Trichomycetes*.

Kolle and Hetch (1911) recognize the *fission fungi*, divided into Cocci, Bacilli and Spirilla, and the *fission algae* including the sulfur bacteria, the iron bacteria and the *Streptothricaceae*.

Schneider (1912) divides the *Schizomycetes* into seven coördinate families, *Coccaceae*, *Bacteriaceae*, *Spirillaceae*, *Spirochaetaceae*, *Mycobacteriaceae*, *Chlamydobacteriaceae* and *Beggiatoaceae*.

Benecke (1912) recognizes two orders, *Haplobacterinae* with six families and *Desmobacterinae* with one family.

Engler (1912) differentiates the *Eubacteria* with six families and *Thiobacteria* with two families.

Vuillemin (1913) insists that the group *Microsiphonees* including the higher filamentous forms should be separated from the *Schizomycetes*.

A review of these groups show them to be based in the various classifications upon different characteristics. Unfortunately but

few authors have included all the forms commonly grouped with the bacteria, so that the classifications do not coincide in the types considered.

Some authors (as Cohn (1872), Zopf (1883), Flügge (1886), De Toni and Trevisan (1889), Ludwig (1892), Hueppe (1895), Migula (1897), Chester (1901), Kendall (1902), Schneider (1912)) based their primary groups upon the shape of the bacterial cells, usually recognizing filamentous forms as one of the groups. This in the various classifications gave rise to from three to six or eight coördinate groups.

A few authors (De Bary (1884), Hueppe) have differentiated organisms as endosporous and arthrosporous. A fuller knowledge of the life history of the various microörganisms has led to an abandonment of this grouping as not tenable.

The work of Winogradsky (1888) and others on the sulphur bacteria has led some authors (as Migula (1900), E. F. Smith (1905) Engler (1912)) to recognize the true bacteria (*Eubacteria*) and the sulphur bacteria (*Thiobacteria*), as primary coördinate groups.

The studies of Thaxter (1892 *et al*) has induced a few authors to recognize the Myxobacteria as an important group. Among such are Lotsy (1912), Smith (1905) and Engler (1912).

The differentiation of bacteria on the basis of true filament production (not merely chain formation) was first suggested by Cohn (1875) and has been used as a primary grouping by De Toni and Trevisan (1889), Fischer (1895), Lehmann and Neumann (1896), Fischer (1903), Lotsy (1907), Heim (1911), Benecke (1912) and Vuillemin (1913). In many respects this has appealed more strongly than any other basis of differentiation to a majority of taxonomists in recent years.

The suggestion of Jennings that bacteria should be separated into those which divide longitudinally and those which divide transversely has never met with favor. The order *Paraschizae* was based upon Metchnikoff's (1888) genus *Pasteuria* which is generally regarded as not belonging with the bacteria and the genus *Astrobacter* described by Jennings based upon stained mounts from stagnant water prepared to show the flagella of

Spirillum undula. This latter genus *Astrobacter* was never recognized in a living condition. The evidence that bacteria were actually observed is by no means conclusive. Jennings' grouping can therefore scarcely be regarded as valid. However, it is possible that the differentiation might be revived for the separation of the true bacteria and the forms more closely related to the protozoa.

The evidence brought forward by Schaudinn (1905) and his followers in the study of the spirochetes makes it probable that they should either be located definitely among the protozoa or as a main group among the bacteria showing many intermediate characters.

The work of Ellis (1907) and of Molisch (1910) has emphasized the individuality of the iron bacteria, justifying certain of the earlier writers in regarding them as one of the principal groups of the Schizomycetes.

Jensen's classification of bacteria into *Cephalotrichinae* and *Peritrichinae* has much that is attractive, but a careful study of his proposed groupings shows that they are not well defined.

It would seem from a review of the literature and a consideration of the characteristics of the organisms that the following principal groups may be recognized among the bacteria or *Schizomycetes:*

1. The true bacteria which include the forms most commonly studied in the laboratory; they are probably more primitive than other more highly differentiated groups.

2. The thiobacteria characterized by certain relationships to sulphur. They all grow best in the presence of hydrogen sulphid, and always contain sulphur granules or bacteriopurpurin or both.

3. The myxobacteria showing a pseudoplasmodial stage, and fruiting stages resembling in some respects those of the slime molds.

4. The iron bacteria, usually sheathed, frequently growing in water containing iron and with a deposit of iron oxid in the sheath; typically water forms without true branching, showing relationships with the algae.

5. The thread bacteria or ray fungi which show a filamentous form, frequently with true branching. Not water forms. As a group intergrading with the fungi.

6. The spirochetes, slender organisms usually spiral and frequently flexuous, showing many characteristics relating them to the protozoa.

In each of the groups here indicated, except the true bacteria, there is a definite specialization in some direction; forms which are close to the true bacteria are to be found in each group, while others within the group may show an approach to the algae, the fungi or the protozoa.

If the group *Schizomycetes* is to be regarded as a class, the subgroups should receive ordinal names. They may be termed the *Eubacteriales*, *Thiobacteriales*, *Myxobacteriales*, *Chlamydobacteriales*, *Actinomycetales* and *Spirochaetales*, respectively.

The following key to these orders gives the most striking of the differential characters.

A. Plant-like in the principal characters, not protozoan like, cells never slender, flexuous spirals; cell division never longitudinal.
  I. Not producing a pseudoplasmodium during the vegetative stage; without a highly developed, cyst-producing, resting stage.
    a. Containing neither granules of free sulphur, nor bacteriopurpurin, nor requiring the presence of hydrogen sulphid for the best development.
      1. Not typically producing filaments as a regular growth form, though chains of cells may be developed. Conidia not developed, spores when formed are endospores.
      Order I. *Eubacteriales*
      2. Typically producing true filaments as a regular growth form. Conidia may be developed, but never endospores.
        (a) Alga-like, typically water forms. Filaments never showing true branching; false branching may be present. A sheath usually evident, and usually impregnated with iron.
        Order II. *Chlamydobacteriales*
        (b) Mold like, not typically water forms, nor with the sheath impregnated with iron. True branching often evident.
        Order III. *Actinomycetales*
    b. Cells typically containing either granules of free sulphur or with bacterio-purpurin or both, usually growing best in the presence of hydrogen sulphid.
    Order IV. *Thiobacteriales*

II. Cells united during the vegetative stage into a pseudoplasmodium which passes over into a highly developed, cyst-producing, resting stage.

Order V. *Myxobacteriales*

B. Protozoan-like in many characters. Cells usually relatively slender flexuous . spirals; multiplication of cells apparently by longitudinal division in some types, by transverse division in others, or both.

Order VI. *Spirochaetales*

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THE EVOLUTION AND RELATIONSHIP OF THE GREAT GROUPS OF BACTERIA

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It is a difficult feat of the imagination to reconstruct the path of evolution of any group of organisms, especially that of the bacteria. In all cases certain difficulties are encountered; but, whereas among the higher organisms one is troubled by the lack of transitional types, the main difficulty among bacteria lies in the superabundance of intermediate strains. Moreover, while, among the former, one is guided chiefly by gross structural distinctions, among the latter, one has to rely to a great extent on the finer biochemical and metabolic differences. Yet an attempt to trace the evolution of these simple cells may well lead to a clearer conception of the character of the organisms and the nature of their adaptation to a saprophytic, parasitic or pathogenic mode of life.

Impossible as it may be to say with certainty when and how bacteria originated, the evidence, little as it is, seems to point to these minute unicellular organisms as among the most primitive of living forms.[1] The simple structure of the cell and the absence of a formed nucleus mark them as possibly the simplest type of cell. The ability of some types to subsist on simple inorganic substances (CH_4, NH_3, and CO_2) without the aid of sunshine, and the sensitiveness of all bacteria to the action of sunlight suggest their existence on this planet prior to the appearance of plant life or the penetration of the rays of the sun

[1] For a full discussion of the subject see the article by Henry Fairfield Osborn on The Origin and Evolution of Life upon the Earth, Scientific Monthly, **3**, 1916.

through the volcanic vapors. Finally, Walcott's discovery of bacteria closely resembling our nitrogen fixers of the soil in Algonkian deposits is an added proof of the primitiveness of these microbes.

The intimate dependence of both plants and animals on bacteria and their activities tends to strengthen the conviction that these microorganisms must have preceded the others. That plants are directly dependent on bacteria is well known. Plants cannot subsist without nitrates and soluble phosphates and both these substances, unless provided as chemical fertilizers, are rendered available in the soil through the action of bacteria. In the arid regions where plant life is absent Lipman found that bacterial life was also absent and where plants do exist they send their rootlets into that part of the soil where bacterial activity is most abundant. That animals cannot develop in the absence of microbial life is well illustrated by the futile attempts to cultivate amoebae without the aid of living or dead bacteria. Recently this relationship was even more strikingly emphasized by the experiments conducted by Loeb who, though successful in hatching adult flies from sterile eggs in sterile media, has so far failed to carry them through the second generation on sterile fruit, although fruit is the normal food of the fly in question.

The story of the evolution of bacteria, like that of the higher forms, must be from the simple to the complex—but along a different line. Microbial evolution must be conceived as an adaptive development in the direction of mobilizing more and more molecules (enzymes) to enable the cell to utilize the more and more complex nitrogen and energy-yielding substances which accumulate in the soil as a result of their own and other biotic activity. It is the probable course of these adaptive modifications that I shall attempt to trace.

It is well known that the smaller the body the greater, proportionately, are its energy requirements. Bacteria need only minute amounts of nitrogenous food but require a relatively enormous quantity of energy-yielding (carbon) compounds. It is, therefore, reasonable to expect the trend of bacterial evolution to have been in the direction of the utilization of a

wider range of energy-yielding substances. We know that most saprophytic bacteria (and even many of the parasitic forms) when supplied with a suitable carbon compound—usually glucose—can utilize either NH_3 or NO_3 nitrogen. At the same time we find great diversity among them in their ability to use various carbon compounds. It is not merely an accident that bacteriologists have in recent years turned to carbohydrates and related substances for the differentiation of bacterial types. It is in this direction that greatest differentiation exists and this is most probably the path of early evolution.

With the increasing deposits of decaying nitrogenous matter a new line of adaptation began. Accordingly we find that as we go higher in the scale of bacterial life the power of utilizing complex nitrogenous compounds (the amino-acids, the proteoses and even simple and complex proteins) comes more and more into play.

The probability that the course of bacterial development was as suggested is strengthened by other evidence. The sparing effect that carbohydrates, (Kendall) alcohols (and, in all probability, other energy-yielding substances) exert on the utilization of nitrogenous compounds, or, in other words, the preference shown by bacteria for carbonaceous food; the inhibitive influence that sugars have on the secretion of proteolytic enzymes (Kligler, Kendall and Walker); the preference shown by certain types of bacteria for the simpler carbohydrates as shown in the metabolic gradients, both among the colon-typhoid group and the cocci; the ability of a large number of bacteria to utilize a wide range of energy-yielding substances from formic through the other fatty acids, the triatomic alcohol and more complex sugars and alcohols; the ability of a considerable number of common bacteria to obtain their nitrogen readily from ammonia; and, finally, the inability of many of the common bacteria to utilize proteoses and complex proteins, though they can use simple amino-acids—all indicate an earlier and more deep-seated development of fermentative powers, followed by later adaptation in the direction of the elaboration of pepto- and proteolytic enzymes.

This evolutionary process is observed in both group and

species development. The colon-typhoid bacteria and the streptococci form distinctive fermentative groups, while the aerobic and most of the anaerobic spore-bearing bacteria constitute distinctive proteolytic groups. At the same time there are members of the former groups that secrete proteolytic enzymes (*B. cloacaė*, proteus forms, staphylococci, micrococci) while some of the aerobic and practically all the anaerobic spore-formers are capable of at least fermenting glucose, which, as is the case in the fermentative group, exerts a definite sparing effect on the secretion of proteolytic enzymes. The dominant and subordinate activities of these two groups indicate clearly, however, the sequence of their development.

Nor are these functional changes entirely unaccompanied by structural modifications. In some cases, to be sure, such differences are not discernible; in others, however, they are more marked. These structural differences are brought out in the behavior of the cells to stains—the ability of the cell to bind iodine, due, probably, to variations in the lipoidal content; the presence of definite granular (chromatin) material; the presence of branching and, finally, the presence of spores. The simple fermentative types are gram negative, show no granular structure and bear no spores. The diphtheria and tuberculosis groups are gram positive, have a definite granular structure, possess little fermentative power but definite proteolytic activity, the nature of which is not yet known. Finally, we have the usually gram positive spore-bearing bacteria with distinct proteolytic tendencies.

In the course of their evolution, with the advent of plant and animal life, some of these micro-organisms have become adapted to a parasitic existence often to the detriment of their host. With this adaptation to the host there followed, usually, a loss or modification of function but a general relationship and resemblance to saprophytic ancestors remained. As a result we find in almost every group of bacteria (as in the case of other parasitic plants and animals) both parasitic and pathogenic, as well as saprophytic forms. In all these cases the presumption naturally is that the parasitic and pathogenic types are off-

shoots from the saprophytes and that the main line of evolution was carried on by the saprophytic bacteria in response to a particular selective environment.[2]

Glancing over the range of microbial life we find purely oxidative processes associated with the simplest and probably the most primitive bacteria—the prototrophic forms. These organisms derive their food from simple inorganic substances, the oxidation of C, N, S and Fe compounds furnishing the energy. It is not at all improbable that the four groups of oxidizers—the carbon, sulphur, iron and nitrogen oxidizers, respectively—arose at about the same time, independently of one another. Nevertheless it seems fairly certain from the important part played by carbon compounds in the vital activities of our common bacteria, especially as a source of energy, that the carbon oxidizers are the forerunners of the bacteria of today. Starting, therefore, with methane, the simplest carbon compound, at the base line, the oxidizers of CO would follow and from them would arise in succession those organisms capable of utilizing CO_2, formic acid, acetic acid, alcohol, etc. Since the ammonia and nitrite oxidizers (or nitrifiers) also assimilate large amounts of carbon-dioxide, (Jensen) they would seem to fall in line along with those organisms capable of obtaining their energy from carbon-dioxide.

[2] Whether these selective influences were exerted on slight cumulative variations or on mutations does not effect the general argument. Examples of what may appear to represent one or the other mode of development exist. The slow transition from the aerogenes type through the *B. cloacae* to *B. proteus* may be an instance of gradual selection, while the abrupt transition from the non-spore-bearing to spore-bearing bacteria may well be cited as an instance of mutation. Fluctuating variations are abundant among bacteria due to the simple character of the cell and its intimate relation to the environment which renders it highly susceptible to external influences. As yet, true mutations have been definitely shown to occur only in a few isolated instances, where single cell cultures were used. But even in these cases the possibility of gradual selection cannot be excluded, since the newly acquired property (usually the power to ferment a given carbohydrate) manifests itself only after the colony is a few days old, in other words, after numerous generations have arisen from the single parent. In connection with the general thesis it is interesting to note that the new character acquired by these mutants is usually one already existing in some ancestral type of the group.

All these bacteria are strictly aerobic, depending on the oxygen of the atmosphere for their oxidative processes. At about this stage of evolution, however, with the accumulation of stable oxides, a new branch developed, differing from the main trunk in its power of utilizing combined oxygen for intracellular combustion. The prototrophic denitrifying bacteria described by various authors are most probably the progenitors of this group.

This radical deviation from type gave rise to a new and distinct line. On the one hand we have the strictly aerobic, oxidative, usually gram positive, non-fermentative organisms with proteolytic tendencies and a complex cell structure—the nitrogen fixers (possibly the fluorescent forms fall in here), the diphtheria, tuberculosis and actinomyces groups; on the other hand we have the facultative aerobic, reducing (i.e., obtaining their oxygen from an oxy-compound), usually gram negative strongly fermentative simple cells—the aerogenes, colon, typhoid, proteus, septicemia groups. The striking and fundamental morphological chemical and immunological differences existing between these two groups leave no doubt that they represent two distinct lines of development. Moreover the relative stability and definiteness of type characterizing the first group are indicative of a more remote origin while the relative instability and the large number of transitional forms found in the second group point to a more recent development. Recency of origin and instability of type run a remarkably parallel course.[3]

The line of descent from the prototrophic denitrifiers is entirely clear. The capsulated aerogenes group has in the last two years been definitely shown to be a saprophytic soil and grain type. Its relation to the so-called prototrophic denitrifiers, though not altogether established, is borne out by its power to live in simple inorganic media and under certain conditions, even to fix atmospheric nitrogen (Löhnis). With more detailed study of soil bacteria it may even be possible to find intermediate forms. From this type two groups arise—one essentially parasitic, the other saprophytic. The parasitic

[3] The tuberculosis and dysentery groups respectively are examples of the two classes.

branch starts with the fermentative gas-producing *B. coli* types, which lose their fermentative character with increasing parasitism and develop intermediate forms ranging from the fermenters with gas through fermenters without gas to non-fermenting types. This leads through the typhoid and dysentery bacilli to the septicemia group.

The second or saprophytic branch evolves by way of *B. cloacae* (almost identical with *B. aerogenes*, except for lack of capsule formation and the added variable power to secrete a tryptic enzyme) to the proteus group, which possesses more intense proteolytic properties developed at the expense of the fermentative powers. The *B. vulgaris* still actively ferments glucose and sucrose though, like the paratyphoid, it has lost the power to ferment lactose. Being saprophytic in character, however, it has developed the property (first manifested by the *B. cloacae*) of actively secreting a tryptic enzyme. This is manifestly the first type possessing both marked fermentative and proteolytic characters. From this type two lines of development are possible,—one, leading to the complete loss of fermentative powers (which appears to be the tendency in the whole group) and consequently a condition of more or less strict aerobiosis; the other, retaining both characters but undergoing other modifications.

These two lines are represented by the aerobic and anaerobic spore-bearing bacilli, respectively. Just how and when spores evolved is, of course, impossible to say but there are many indications of the relationship between the spore-formers (aerobic as well as anaerobic) and the *B. proteus*. The relationship with the former is shown by the existence of proteus-like, non-fermenting, strictly aerobic but, as yet, non-spore-forming bacilli which have been repeatedly reported by various authors. These are all soil forms. The kinship with the anaerobes is indicated by the constant association of the proteus bacilli with putrefactive processes; the inability on the part of the anaerobes (with the exception of *B. welchii*) to ferment lactose while still retaining the power to ferment glucose and to some extent sucrose; and, finally, by the marked acid hydrolysis of pro-

teins manifested by both groups of organisms. The transition from facultative aerobiosis to obligatory anaerobiosis is not at all unusual among bacteria. Different degrees of sensitiveness to oxygen tension are manifested by closely related organisms in different groups (e.g., the cocci, the diphtheroids), and it is readily conceivable that an organism subjected to prolonged existence under anaerobic conditions would develop a high degree of sensitiveness even to traces of free oxygen.

A third group of bacilli, which probably had its origin in the proteus family, is that of the pigmented bacteria, including *B. prodigiosus*, *B. rubrum*, etc. Like the proteus forms they are gram negative, peritrichic, liquefying bacteria, some of them also producing gas from glucose. Pigmentation is often found among the cloacae forms and in our collection there are yellow and red pigment producers isolated from water, both of which otherwise behave like typical *B. coli*.

Returning again to the aerogenes group one finds many striking resemblances between these organisms and the capsulated chain-forming cocci. The similarity in the character of the capsule (solubility in acetic acid); the launcelate shape of the cell; the power of both to ferment inulin; their localization in the same organs causing similar disturbances; their active fermentative properties point to a rather close relationship. The fact that the chain bacteria are gram positive indicates a radical structural modification analogous to that in the spore-forming bacteria. Here, again, a comparatively recent development is indicated by the marked instability of types. The non-capsulated streptococci are obviously related to the pneumococci while the milk streptococci are but little removed from the aciduric lactic acid bacilli, usually found in milk and milk products.

While the course of the development of this large branch, composed of the so-called reducing bacteria can be traced with a fair degree of certainty, the path of descent of the main trunk consisting of the strongly aerobic and more complex forms is not quite as clear. The uncertainty regarding the latter group is attributable to at least two factors. It is due partly to the

fact that the metabolic activities of these organisms have not been studied as carefully and partly to the fact that, being the older and more stable group, many of the linking intermediate forms have disappeared and only the dominant types have remained. But even though the details remain somewhat obscure the general relationship is very clearly indicated. The saprophytes from which our second main stem arose are the nitrogen fixers of the soil (azotobacter), powerful oxidizers, oxidizing nitrogen to nitrates and ethyl alcohol to acetic acid (Jensen). Their nearest relative is the nitrogen fixer of the legumes which may really be considered a plant parasite localizing in the root of the plant and producing a reaction similar to that caused by the tubercle bacillus in the resistant animal body. As a parallel offshot to the *B. radicicola* we have the saprophytic acid fast bacteria (associated usually with grass), and their parasitic relatives, the various types of the tubercle bacillus. Recently the relationship between the acid fast forms and the actinomyces has been clearly established (Claypole). In this latter group, also, there are the soil saprophytes, associated with grass digestion (Conn), as well as human and animal parasites. Arising somewhere from the acid fast group there is the specific parasitic diphtheroid group which has been found nowhere (as far as I know) outside of the animal body. As parasites these are widely distributed in animal tissues, though, up to the present, only the diphtheria bacillus has been found to be definitely pathogenic. The relation of the diphtheria bacillus to the sporothrix is suggested by transitional forms found in the human mouth (Kligler). These organisms are highly pleomorphic showing thread, coccoidal and diphtheroid forms. They are fermentative, aerobic types. The oral cavity seems to be a favorite locality for the whole diphtheroid group.[4]

While there is an apparent gap between the azotobacter and the acid fast bacilli, there are still many points of similarity. The acids fasts can thrive on a simple medium with ammonium

[4] This localization may account for the fact that this group differs so markedly from the other members of the class in its power to ferment carbohydrates, particularly glucose and maltose.

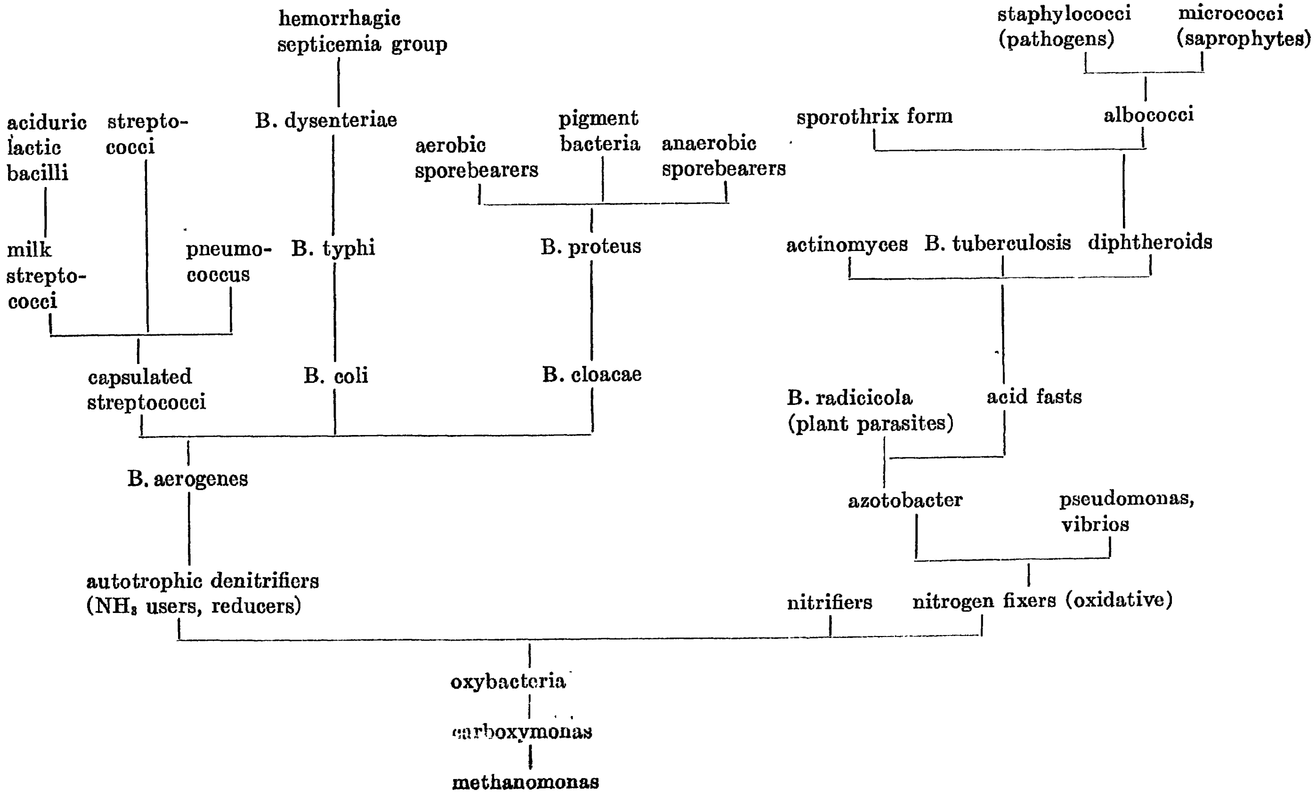
compounds as a source of nitrogen and glucose as a source of energy, while recent investigation has brought out the fact that the azotobacter can assimilate free nitrogen more readily if glucose and a small amount of ammonia are supplied. The nodule nitrogen fixers with their branching cells and localized infective power with nodule formation show even more marked resemblances to the acid fast bacteria. The whole group is characterized by absence of fermentative properties (except the diphtheroids), strict aerobiosis, a peculiar nitrogen metabolism the nature of which is not at all clear (this applies to all the members of the group), the gram positive reaction, a complex granular structure of the cell with a tendency towards branching and finally the peculiar local reaction often nodular in character which is produced by the pathogenic varieties.

The general parasitic character of the white staphylococci, their moderate fermentative character, lack of reducing and liquefying properties, together with their gram positive reaction and the diphtheroid character of their growth link them to the diphtheroid bacilli. From white cocci there arise the more highly parasitic and pathogenic staphylococci on the one hand and the saprophytic micrococci on the other. A group of transitional saprophytic albococci has been shown to exist (Winslow), while yellow pigmentation is often observed among the diphtheroids.

There is one group of organisms—the fluorescent forms—the position of which is hard to determine. We find soil nitrogen fixers that produce fluorescence. On the other hand the fluorescent bacteria are strong ammonifiers, though they, like the nitrogen fixers, are aerobic and non-fermenters. Their simple structure, lophotrichic flagella and gram negative reaction mark them as simple bacteria. Perhaps they occupy an intermediate position. For the present we may place them with the oxidative bacteria.

The vibrios are so closely akin to the fluorescent (or pseudomonas) bacteria that their place in the scheme outlined depends on the position assigned to their related group. In fondness for oxygen, general proteolytic character, and in structure and

SCHEMATIC OUTLINE OF THE PROBABLE RELATIONSHIP OF THE VARIOUS GROUPS OF BACTERIA



appearance of cell these groups resemble each other so closely as to make it at times difficult to differentiate them from one another.

The accompanying diagram attempts to present in a schematic way by no means rigid or exact the probable lines of evolution of the different groups of bacteria. Much of it may be faulty and it is obviously incomplete, but in its essential outline it seems to me to be correct and helps to show these organisms in their probable relationship to one another as members of a single if diversified order.

A STUDY OF FIVE MEMBERS (OR SO-CALLED SPECIES) OF THE SEPTICEMIA HEMORRHAGICA (PASTEURELLA) GROUP OF ORGANISMS WITH SPECIAL REFERENCE TO THEIR ACTION ON THE VARIOUS CARBOHYDRATES

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Many species of animals are susceptible to the disease known as septicemia hemorrhagica or pasteurellosis. This disease has been reported in cattle, horses, reindeer, buffalo, fowls, rabbits, and pigs. When an organism has been isolated from an animal infected with septicemia hemorrhagica, it has usually been named according to the animals from which it has been isolated, as *Bact. bovisepticum*, *Bact. avisepticum*, or *Bact. Renntierpasteurella.* It is generally considered that these organisms are similar in morphological characters and in many of their biological properties.

The object of the present work was to determine whether the members of this group could be differentiated by their cultural properties, especially by their biochemical action on the various carbohydrates. As the investigation has been limited to a study of biochemical characters, the summary of literature mentions only those works which have taken up these characters in detail.

Table 1 gives a summary of the action of the different members of this group on the various carbohydrates, as determined by certain investigators.

Table 2 shows the production of indol and phenol as recorded by various bacteriologists.

TABLE 1

Action of members of the Septicemia Hemorrhagica group on the various carbohydrates as reported by Magnusson (1914), Vourloud (1907) and Schirop, (1908)

	FRUCTOSE	GALACTOSE	GLUCOSE	MANNOSE	SORBOSE	ARABINOSE	XYLOSE	RHAMNOSE	LACTOSE	MALTOSE	SUCROSE	RAFFINOSE	AMYLOSE	DEXTRIN	INULIN	GLYCERINE	ERYTHRIT	ADONIT	DULCIT	MANNIT	SORBIT	AMYGDALIN
1. Magnusson																						
Renntierpasteurella..	+	+	+	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+	+	
Bact. bovisepticum..	+	+	+	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+	+	
Bact. avisepticum....	+	+	+	+	−	−	−	−	+	−	+	−	−	−	−	−	−	−	−	+	+	
2. Vourloud																						
Bact. avisepticum....	+	−	+			−	−	−	−	−	+			−	−	−	−	−	−	+	+	+
3. Schirop																						
Schweineseuche......	−	tr.	+			−				−	+	−					−	−		−	+	
Geflügecholera........	+	tr.	+			−				−	+	−					−	−		+	+	

\+ Acid production.
− No acid production.
tr. Trace of acid.

TABLE 2

Variations in the production of indol and phenol as reported by certain authors

AUTHOR	ORGANISM	INDOL	PHENOL
Flügge (1896).............	*Bact. suisepticum*	−	−
	Bact. buffaloseuche	+	−
	Bact. Renntierpasteurella	+	+
Buchanan (1911)...........	*Bact. avisepticum*	+	+
	Bact. suisepticum	+	+
	Bact. bovisepticum	+	+
Lignières (1910).............	(Invariable characters)	−	−
Magnusson (1914)..........	*Bact. Renntierpasteurella*	−	
	Bact. avisepticum	+	
Moore (1916)..............	*Bact. avisepticum*	+	
	Bact. cuniculicida	+	+
	Bact. suisepticum	+	
	Bact. bovisepticum	+	
Smith (1891).................	Bacteria of swine plague	±	+

M'Gowan and Wang (1915) carried out a very extensive study of *Bact. avisepticum* with special reference to the change in the biochemical action of the culture as its virulence was increased by passing through a series of guinea pigs and rabbits. The results of their investigations show that the culture, which originally only produced acid from certain carbohydrates was so changed after passage through seven guinea pigs that it produced acid and gas. They also found that cultures isolated from different sources varied to the extent that some produced only acid while others produced both gas and acid.

The methods used in the investigation here reported are as follows: The acid fermentation of the different carbohydrates was determined in media prepared from sugar free meat infusion bouillon with the reaction adjusted to about +0.3, Fuller's scale. To this sugar free bouillon 1 per cent of the various carbohydrates was added. An increase in acidity was very marked in some of the carbohydrate bouillons after sterilization. A number of tubes of the same carbohydrate bouillon were inoculated with the same culture and incubated at 37°C. At various intervals, as indicated in the tables, tubes were removed and titrated. Other tubes which were not inoculated were titrated to serve as checks. The results of the titrations are expressed in the number of cubic centimeters of twentieth normal solution[1] required to neutralize 5 cc. of the culture. A minus (−) indicates an alkaline reaction, and no sign indicates an acid reaction in the culture.

The ten cultures used in this work are designated by the names given them by the bacteriologist who isolated the cultures. These had been held at stock cultures for varying lengths of time. The source of the cultures is as follows:

Bact. bovisepticum was isolated August, 1914, from a case of septicemia hemorrhagica in a cow.

Bact. avisepticum, J1 and J2, were isolated about 1911 from cases of chicken cholera.

Bact. suisepticum was isolated about 1914 from the lung of a pig.

[1] N/20 NaOH or N/20 HCl.

Bact. Kälberpasteurella A-B and *Bact. Renntierpasteurella* A-B-C, were received from Magnusson about February, 1915.

Bact. septicemia hemorrhagica was isolated about 1910 from a cow.

Table 3 gives the detailed results of titrations of one of the cultures studied. The same number of titrations were made

TABLE 3

Action of Bact. suisepticum (no. 1) upon twenty carbohydrates

DAYS INCUBATED	GLUCOSE		LACTOSE		SUCROSE		MANNIT		DEXTRIN		INULIN		GALACTOSE	
Check	0.7	0.7	0.7	0.6	0.4	0.4	0.5	0.5	0.7	0.8	0.7	0.7	0.9	0.9
1	1.5	1.5	0.6	0.5	2.1		1.8	1.7	0.8	0.8	0.7	0.7	1.1	1.0
2	2.1	2.2	0.5	0.7	2.1	2.2	1.9	1.9						
3	2.2	2.2	0.5	0.5	2.0	2.1	2.0	2.0	0.7	0.8	0.4	0.4	2.2	
4	2.2	2.1	0.6	0.4	2.2	2.2	1.9	1.9						
5									0.5	0.4	0.2	0.1	2.2	2.5
7	2.3	2.1	0.4	0.4	2.1	2,3	2.1	2.0						
10	2.6	2.5	0.0	−0.1	2.4	2.3	2.2	2.2	0.1	0.3	−0.4	−0.3	2.2	2.3
30	2.4	2.6	−0.1	0.1	2.2	2.3	1.9	2.1	−0.2	0.1	0.8	−0.4	2.5	2.6
DAYS INCUBATED	LEVULOSE		GLYCERINE		RAFFINOSE		AMYGDALIN		MANNOSE		MALTOSE		ARABINOSE	
Check	0.8	0.8	0.4	0.3	0.3	0.3	0.4	0.4	0.7	0.7	−0.3	−0.3	0.2	0.2
1	0.9	1.0												
2			0.3	0.3	0.4	0.3	0.3	0.3	2.6	2.6	0.3	0.2	0.2	0.2
3	2.3	2.3												
4														
5	2.3	2.2	−0.1	−0.1	−0.1	−0.1	0.2	0.0	2.7	2.8	−0.3	−0.3	0.2	0.2
7														
10	2.4	2.4	−0.1	−0.1	−0.3	−0.2	−0.3	−0.3	3.4	3.3	−0.6	−0.4		
30	2.2	2.3	1.3	−0.1	−0.4	0.9	0.3	0.9	2.8	2.8	−0.6	−0.4		
DAYS INCUBATED	SALICIN		XYLOSE		DULCIT		ISODULCIT		ADONIT		ERYTHROL		MILK	
Check	−0.3	−0.3	0.5	0.5	−0.3	−0.3	−0.3	−0.3	0.2	0.2	0.2	0.2	1.0	1.1
1														
2	−0.3	−0.3			−0.3	−0.2	−0.1	−0.2	0.2	0.1	0.1	0.1	1.1	1.0
3							−0.2	−0.3						
4														
5	−0.3	−0.1	1.9	1.6	0.1	0.1	−0.3	−0.3	−0.2	−0.2	−0.2	−0.2	1.1	1.1
7														
10			1.3	1.9			−0.4	−0.4	−0.2	−0.3	−0.2	−0.3	1.3	1.2
30			0.2	0.2					0.6	0.7	0.3	0.3	1.4	1.3

upon each of the other nine cultures. The cultures were all so similar that the general results only are reported in table 4.

Cultures of *Bact. avisepticum* (J2) and *Bact. Kälberpasteurella* (*Magnusson A*) were inoculated into rabbits. The rabbits died within twenty-four hours, and pure cultures of the organisms were recovered from the heart, liver, spleen, and kidney. Sub-

TABLE 4

	GLUCOSE	LACTOSE	SUCROSE	MANNIT	DEXTRIN	INULIN	GALACTOSE	LEVULOSE	GLYCERINE	RAFFINOSE	AMYGDALIN	MANNOSE	MALTOSE	ARABINOSE	SALICIN	XYLOSE	DULCIT	ISODULCIT	ADONIT	ERYTHROL
1. *Bact. suisepticum* (no. 1)...	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−
2. *Bact. bovisepticum* (Baker).	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−
3. *Bact. avisepticum* (J2)......	+	−	+	+	−	−	+	+	−	−	−	+	−	+	−	+	+	−	−	−
4. *Bact. avisepticum* (J1)......	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−
5. *Bact. Kälberpasteurella* (A).	+	+	+	+	−	−	+	+	−	±	−	+	−	−	−	+	−	−	−	−
6. *Bact. Kälberpasteurella* (B).	+	+	+	+	−	−	+	+	−	±	−	+	−	−	−	+	−	−	−	−
7. *Bact. Renntierpasteurella* (A)....................	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−
8. *Bact. Renntierpasteurella* (B)....................	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−
9. *Bact. Renntierpasteurella* (C)....................	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−
10. *Bact. septicemia hemorrhagica* (bovine)............	+	−	+	+	−	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−
After recovery from rabbit																				
3. *Bact. avisepticum* (J2)......	+	−	+	+	−	−	+	+	−	−	−	+	−	+	−	+	+	−	−	−
5. *Bact. Kälberpasteurella* (A).	+	+	+	+	−	−	+	+	−	+	−	+	−	+	+	+	−	−	−	−

\+ Acid production.
± Slow and weak acid production.
− No acid production.

cultures were made in agar plates, litmus milk, and lactose bouillon fermentation tubes. No indication of contamination was apparent in these cultures nor was any found by microscopic examination. The action of these two cultures upon the carbohydrates after recovery from the rabbit is recorded at the bottom of table 4.

All ten cultures produced indol and phenol in sugar free infusion bouillon. The organisms gave a scant growth in 1 per cent peptone solution, but it was insufficient for a positive test for indol, phenol, or ammonia. However, the growth in the peptone solution was sufficient to demonstrate the reduction of nitrates.

Gelatin was not liquefied by any of the cultures within thirty days at 20°C. Gelatin cultures, after being incubated at 37°C. for thirty days, solidified on cooling.

Litmus milk and plain milk were apparently unchanged by any of the cultures. Titrations, however, show that the two lactose fermenting cultures (*Bact. Kälberpasteurella* A-B) produced a small but distinct increase in acidity. The probable reason why apparent changes are not produced in milk is the poor growth in any medium having a high acid reaction. Difficulty was encountered in obtaining growth in bouillon when the acidity was 0.7 or 0.8 (Fuller's scale). The normal reaction of even very fresh milk exceeds this.

At the beginning of this work, each of the ten cultures was inoculated into glucose, lactose, sucrose, and mannite bouillons in Smith fermentation tubes. No gas was produced in any case. It was assumed from this and from the records of previous investigations that gas was not produced in these carbohydrates. The titrations here recorded were not made from fermentation tubes.

Sorbit could not be obtained. Some of the other chemicals, as dulcit, were so expensive that very few titrations were made with them (see table 3).

Table 4 gives a summary of the action of the cultures upon the various carbohydrates.

In general, the results of this investigation correspond to those of Magnusson. Magnusson, however, reports acid fermentation of lactose by *Bact. Renntierpasteurella*, *Bact. bovisepticum*, and *Bact. avisepticum*, while in this investigation *Bact. Kälberpasteurella* only produced acid from lactose. *Bact. Kälberpasteurella* and *Bact. Renntierpasteurella* were received from Magnusson.

The results here reported show very marked uniformity in the biochemical action of all cultures studied, while M'Gowan and Wang found wide variations in cultures isolated from different sources. In this investigation no attempt was made to increase the virulence of the cultures, therefore no direct comparison can be made in this regard.

Table 4 shows acid production from xylose by all cultures studied. My results here differ from those of Magnusson and Vourloud. In this study it was found that xylose greatly increased the acidity of bouillon on sterilization, and the cultures would not grow in this acid bouillon. If the initial reaction of the bouillon was such that the final acidity was not too high, the cultures grew and produced acid. From this it seems possible that differences in technique may account for different results with xylose.

The chief differences in the cultures studied are in the fermentation of lactose and raffinose by the two cultures of *Bact. Kälberpasteurella* received from Magnusson and the fermentation of arabinose and dulcit by *Bact. avisepticum* (*J2*). Since these differences persist after recovery from a rabbit, it is improbable that the cultures were contaminated. Furthermore, the work was repeated, where differences were found, after the cultures had been plated and recovered from the plates. The second results agreed with the first, and cultural and microscopic examination did not disclose contamination.

The tubes incubated for thirty days gave variable results. That is, the duplicates sometimes showed extremely different degrees of acidity or alkalinity. In some instances, the production of acid was marked after thirty days and not at fifteen days. Since the plugs of the tubes incubated thirty days were paraffined while the others were not, the difference in the interchange of air may have modified the action of the organisms. Except in the case of the action of the cultures of *Bact. Kälberpasteurella* on raffinose, nearly the maximum acidity was produced within two days. For these reasons, the titrations made after thirty days incubation were considered unreliable, and, therefore, if an increase in acidity was not found within fifteen

days, the organism was recorded in table 4, as not fermenting the carbohydrate used in the test.

The results of this work show some discrepancies with the findings of previous investigators. These variations may be accounted for by differences in technique or by slight variations in the cultures, caused possibly by different methods of culturing.

The most striking feature brought out by the study of these organisms is that there is a much greater uniformity between the members of this group in their biochemical properties than has been noted in the study of some other groups of bacteria. There seems to be no biochemical basis for designating by different names the five members of this group which were studied.

CONCLUSIONS

1. The members of the septicemia hemorrhagica group studied were practically uniform in their biochemical actions.
2. The passing of an organism through a rabbit did not change its biochemical characters, except to a very slight degree.

The author wishes to acknowledge the assistance of Dr. C. P. Fitch, of the New York State Veterinary College, in suggesting this study and in giving advice in carrying out the investigation. This investigation is a continuation of the work of Dr. Fitch, reported in Report of New York State Veterinary College of 1913–1914.

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THE LARGE NUMBERS OF BACT. ABORTUS VAR. LIPOLYTICUS WHICH MAY BE FOUND IN MILK[1]

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In a recent publication (1916) on the bacterial flora of the normal udder it was shown that a variety of *Bact. abortus* named *lipolyticus* is common in freshly drawn milk. This variety is characterized by the property of hydrolyzing butterfat with the consequent formation of fatty acids.

The greatest number of *Bact. abortus var. lipolyticus* per cubic centimeter of freshly drawn milk reported in that paper was 50,000. The largest percentage of samples plated from one dairy in which this organism and related strains were found was 51. The statement was made that the figures given in the table showing the frequency of *Bact. abortus* in freshly drawn milk were undoubtedly far below the actual numbers present in the milk, because the methods by which *Bact. abortus* could be demonstrated were developed gradually as the work progressed, and were poorly adapted to the growth of these organisms in the early part of the study.

A few weeks ago twenty-three samples of milk were obtained from a dairy farm, and were plated for *Bact. abortus*. It was the same dairy farm from which the samples were obtained which in the earlier study showed *Bact. abortus* in 51 per cent of cases. The methods of plating and subculturing were practically the same as described in the earlier paper, but a more mature experience in recognizing colonies on the plates and in coaxing unwilling subcultures to grow revealed much larger numbers of *Bact. abortus* in the milk than were previously reported.

Bact. abortus colonies developed in seventeen, or 73.9 per cent of the twenty-three samples of milk plated. In the milk from

[1] Published by permission of the Secretary of Agriculture.

one cow there were 112,000 of these organisms per cubic centimeter. If the milk from the 23 cows had been mixed, it would have contained approximately 7000 *Bact. abortus* per cubic centimeter, and this form would have made up about 28 per cent of the total number of bacteria. Cultures from eleven of the samples were studied in detail, and it was found that nine of them were *Bact. abortus var. lipolyticus*, and two resembled the cultures from pathological material.

In all probability these high figures fall short of the actual numbers that were present in the milk, for it is a peculiarity of the *lipolyticus* variety of *Bact. abortus* that it frequently does not grow under conditions which are apparently the same as those under which in another set of plates its development is good.

The sanitary significance of such large numbers of *Bact. abortus var. lipolyticus* in milk is not known, and it is not the purpose of this paper to discuss this question, but to call attention to the fact that there are in milk large numbers of a type of bacteria which do not grow when the milk is plated in the manner practiced in board of health and other laboratories, and which have not been considered in the problem of a pure milk supply.

In the earlier paper it was shown that the fat decomposing variety of *Bact. abortus* will multiply in milk when kept under the conditions to which it is ordinarily subjected in the home before it is consumed, and that the butyric acid formed from the hydrolyzed fat will produce a disagreeable flavor and odorin cream within twenty-four hours, if the initial contamination is high. An initial contamination of 7000 per cubic centimeter is deserving of consideration if an appetizing milk or cream is desired a day or two after it is drawn from the udder.

The thermal death point of *Bact. abortus* is of interest in this connection. Tests have been made, and it has been found that a temperature of 52°C. (125° F.) for thirty minutes, or 63°C. (145° F.) for thirty seconds will kill both the pathogenic and lipolytic varieties in milk cultures. They are therefore readily destroyed by the heat of ordinary pasteurization.

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CREATININE PRODUCTION BY B. COLI AND B. TYPHI

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From the Bacteriological Laboratory of Stanford University

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In a previous paper by the author (Sears, 1916) a brief series of experiments was described which indicated that a number of bacteria are capable of forming in peptone solutions containing glucose a substance or substances giving Jaffe's creatinine reaction and susceptible of accurate measurement in terms of creatinine by the colorimetric method of Folin (1914). The results of these experiments showed an interesting difference between the cultures of *B. coli* and *B. typhi*. These organisms were therefore made the object of the further experiments with which the present paper deals.

A number of strains of *B. coli* and of *B. typhi* were employed in making the tests, and, as the accompanying table shows, the quantitative as well as the qualitative results were entirely consistent. In sugar-free peptone solutions none of the strains of either organism produced a substance giving Jaffe's reaction.

The arrangement of the tests was the same as that described in the author's paper already cited and consisted in inoculating a number of flasks containing 750 cc. of a 2 per cent peptone solution (Witte's), to which approximately 1 per cent glucose had been added, with different strains of the two organisms in question. The flasks were incubated at 37°C. and samples withdrawn at intervals and analysed as indicated. An excess of calcium carbonate (5 grams) was placed in each flask.

In order to relate, if possible, the production of creatinine with the disappearance of sugar, or with acidity, the glucose and acidity were determined in each sample. Benedict's method was used for the former and the usual media titration method for the latter.

It was found that when calcium carbonate was omitted from the flasks the creatinine formation by *B. typhi* was always very slight, rarely reaching a measurable quantity.[1] It is probable, however, that this was due to the prompt inhibition of growth and not directly to the acidity itself since the value of the latter in the cultures containing calcium carbonate remained at a point little, if any, below the maximum value attained by the cultures not containing calcium carbonate. This maintenance of a high acidity in the presence of calcium carbonate has been also observed by Glenn (1911).

ORGANISM	CREATININE (mgms. per 100 cc.)						GLUCOSE USED (mgms. per 100 cc.)						ACIDITY (N/20 NaOH per 5 cc. culture)					
Age of culture, days	1	2	3	4	6	8	1	2	3	4	6	8	1	2	3	4	6	8
B. typhi 65..	3.0	4.1	3.4	3.5	3.5	3.4	183	229	372	478	478	478	2.2	2.5	2.3	2.1	1.9	2.0
B. typhi *Hopkins*..	3.5	5.1	4.0	4.6	4.2	4.2	138	222	266	458	458	458	2.4	2.6	2.4	2.3	1.9	1.9
B. typhi 6...		2.1	3.1	3.0	3.8	3.9	155	210	252	316	365	411	1.8	1.9	1.9	1.9	1.6	1.5
B. typhi 23..		1.9	2.3	2.5	2.8	2.8	146	265	300	300	352	399	2.1	2.3	2.1	2.0	1.8	1.6
B. typhi 8...	2.3	2.4	3.0	3.4	3.5	3.6	191	245	285	309	398	487	1.9	2.2	2.0	1.9	1.9	1.5
B. typhi 16..		2.0	3.0		4.6	5.3	247	323	367		457	495	1.8	2.0	1.7		1.9	1.1
B. typhi 20..		2.1	2.7		3.8	4.9	270	307	347		451	487	1.9	2.2	2.1		1.8	1.2
B. typhi 47..		2.1	2.8		4.1	5.3	237	299	331		443	459	1.9	2.0	2.1		1.8	1.1
B. coli 3....							708	1099					1.7	1.9	2.8	1.8	1.4	1.4
B. coli S....							586	623	707	1099			2.3	3.1	3.0	2.7	2.7	2.7
B. coli D....							384	887					1.9	2.5	2.6	2.3	1.9	1.7
B. coli N....							1009						1.2	1.1	0.9	0.0	1.0	0.5

The qualitative results of these experiments with *B. coli* and *B. typhi* are directly the opposite of those obtained by Zinno (1893) and Antonoff (1906) using sugar-free 2 per cent peptone solutions. Both of these investigators reported the presence of creatinine in *B. coli* cultures on this medium while the cultures of *B. typhi* were free from this compound. Both used Weyl's and Salkowski's tests. In the hands of the author neither of these tests has given consistent results, cultures of different

[1] I wish to express my thanks to Mr. Fred H. Eldred, Washington, D. C., for making the large number of tests which established this point.

strains of the same organisms reacting differently toward them. In view of the well established fact that a large number of substances besides creatinine are capable of giving positive results with these tests (Weyl, 1878, Guareschi, 1888), it is believed that they are worthless for this purpose. Zinno, however, claimed to have proved the presence of creatinine in *B. coli* cultures by preparing crystals of creatinine-zinc chloride from them.

German (1912) also reported the formation of creatinine by *B. coli* grown in 2 per cent peptone solutions. Fitzgerald and Schmidt (1912) were unable to substantiate his findings.

The evidence that the compound present in glucose-peptone cultures of *B. typhi* giving Jaffe's reaction is creatinine is as yet purely circumstantial. All attempts to prepare the substance in a pure state have failed. The color produced on treatment of the culture with picric acid and sodium hydroxide matches exactly that produced on treating the creatinine-zinc chloride standard with the same reagent. Glucose is ruled out as an interfering substance by careful controls, and it is evident from the data given that the acids produced are not responsible for the color reaction. In the absence of direct proof to the contrary, therefore, I believe that it may be stated with a fair degree of certainty that creatinine production in glucose-peptone cultures is a constant quality of *B. typhi*, and that *B. coli* always fails to produce creatinine, in appreciable quantities, in the same medium.

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THE COLORIMETRIC DETERMINATION OF HYDROGEN ION CONCENTRATION AND ITS APPLICATIONS IN BACTERIOLOGY

PART III

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SECTION XIV. ELECTROMETRIC AND COLORIMETRIC COMPARISONS

As indicated in a previous section, the solutions which must be dealt with in bacteriological investigations are diverse indeed and are not subject to a classification which facilitates systematic study of the indicator errors. We have considered it wise, therefore, to omit, for the present, detailed consideration of particular media, and have studied a considerable variety of solutions upon which we have made more than 400 electrometric measurements and many more colorimetric comparisons. These comparisons between the colorimetric and electrometric measurements are given in tables 4 to 19.

In these comparisons we shall consider the hydrogen electrode measurements as the standard, since they were made with an equipment and in a manner which has proved accurate and reliable. The details of the special electrometric methods used will be found in previous papers by Clark (1915 d) and Clark and Lubs (1916). Occasionally an electrometric determinator was made upon a solution not well adapted to hydrogen electrode measurements. Those familiar with the subject will recognize such a solution in Dorset's egg medium, for instance. In one or two other cases electrometric determinations with solutions in a P_H region where their buffer effect is small have been somewhat uncertain. In general, however, the electrometric measurements may be trusted to the second decimal of P_H if it be granted that the correct order of magnitude of liquid contact potentials has been properly determined (Clark and Lubs 1916). Where

the measurements are not to be so trusted the second decimal place will be omitted.

In the colorimetric comparisons we have purposely avoided, in this preliminary survey, some of the best colorimetric methods which we might have applied, such as the use of optical instruments of correct design, and we have used the somewhat wide interval of 0.2 P_H in the standard comparison solutions between which to interpolate. In these interpolations we have estimated only to the nearest 0.1. We have, furthermore, given our attention to colored and turbid solutions rather than to clear, colorless ones. In bringing various media within the range of the several indicators NaOH or HCl has been added, which generally produces a precipitate. This we have not filtered out but have studied the solutions with color and turbidity present. In short, our procedure while carried out with care and with exactly prepared standard comparison solutions has been such as we may reasonably expect the equipment of any laboratory to permit, and upon such material as that to which we must expect it to be applied. The electrometric measurements were made by one of us, the colorimetric measurements by the other and without any exchange of information which could influence the results. Except in two or three cases where obvious mistakes were involved and in two cases in which it was realized that no accurate measurements could be made, no data have been omitted because of disagreement between the values obtained with the two methods. The brackets found in the tables enclose values found for the solutions indicated at the left. Each value represents a determination on a separate sample.

In some instances a sufficient number of determinations were made with one kind of solution to justify a summary. Such summaries are given in tables 4, 5, and 6. In these tables we mean by "average deviation" the value obtained when the electrometric value was subtracted from the colorimetric value in each case and the arithmetical average of the + and − deviations was taken. Thus, in table 4 the average deviation for phenol red, comparator method, is + 0.03, and with dilution method, − 0.01, which means that if the electrometric determi-

nation were $P_H = 7.00$, the average colorimetric value in one case would be 7.03 and in the other 6.99. Since such averages might agree with the electrometric value, although the individual positive and negative deviations were large, we have given the "mean deviation," which is the mean of all deviations neglecting their sign. A $\pm$ following this shows that the P_H values obtained colorimetrically were greater in some cases than the electrometric value and in other cases less. When only $+$ for instance, follows, it shows that the colorimetric P_H in every instance was greater than the electrometric P_H. The maximum and minimum deviations need no explanation except as to sign which follows the order given above.

In tables 8 to 9 we have included under "Remarks" only a rough indication of the depth of color in the solutions studied. As already mentioned, NaOH or HCl was added in many instances and this produced more or less turbidity. Only in the case of extreme turbidity, however, is this mentioned in "Remarks." In order to simplify the tables further, mention of the addition of acid or alkali will be omitted. *It will, therefore, be understood that to bring the* P_H *of certain solutions within the range of the indicators studied HCl or NaOH has been added although not mentioned in the tables.*

In the columns headed "Dilution" the values given were determined after dilution of 2 cc. of the solution to 10 cc. with distilled water. In all but a few cases such determinations were made without a comparator. In the columns headed "Comparator" the values given were determined without dilution and with the aid of a comparator according to Walpole's compensation method (see Journal of Bacteriology, ii, p.118 and 136). When determinations were made with a comparator after dilution of the solution the values given are placed *between* the other columns, unless dilution is mentioned under "Remarks".

There are contained in these tables numerous points which may be emphasized to better advantage in a discussion. A few of the more important will now be considered.

All the indicators studied are reliable for approximate determinations upon a wide variety of solutions, and, if good judgment

is used, they may be depended upon for approximate determinations even when the solution studied is highly colored and turbid. For precise determinations, however, each indicator must be used with caution in particular cases and some of them can not be depended upon.

TABLE 4

Deviations of colorimetric determinations from electrometric determinations of P_H in Dunham's solutions

NUMBER OF DETERMINATIONS	INDICATOR	COLORIMETRIC METHOD	DEVIATIONS OF P_H			
			Average	Mean	Maximum	Minimum
12	Brom phenol blue	Comparator	+0.10	0.10±	+0.16	+0.01
10		Dilution	+0.09	0.11±	+0.24	−0.02
17	Methyl red	Comparator	+0.21	0.21+	+0.35	+0.02
19		Dilution	+0.27	0.27+	+0.45	+0.13
3	Propyl red	Comparator	+0.09	0.10±	+0.15	−0.02
4		Dilution	+0.10	0.11±	+0.15	−0.02
10	Brom cresol purple	Comparator	±0.00	0.04±	−0.10	0.00
4	Brom thymol blue	Comparator	+0.08	0.12±	+0.14	+0.08
5		Dilution	+0.01	0.06±	−0.10	−0.02
14	Phenol red	Comparator	+0.03	0.06±	+0.14	−0.01
8		Dilution	−0.01	0.04±	+0.10	−0.01
8	Cresol red	Comparator	+0.07	0.07+	+0.13	+0.01
10	α-napthol phthalein	Comparator	−0.06	0.06±	−0.14	+0.02
5		Dilution	−0.05	0.05±	−0.11	+0.02
9	Thymol blue	Comparator	+0.07	0.07+	+0.11	+0.05
		Dilution	−0.05	0.06±	−0.15	−0.01

Let us consider each indicator in the order of the range of P_H which it covers.

Thymol blue in its *acid range* seems to be fairly reliable according to the limited number of determinations made with it (see table 8). It is especially gratifying to observe this because hitherto the zone of P_H covered has been without a brilliant and

at the same time reliable indicator. If our few determinations with thymol blue (acid range) are a good indication of its reliability it should be of great use in the study of yeasts,

TABLE 5

Deviations of colorimetric from electrometric values in P_H determinations of beef infusion, 1 per cent peptone broths

NUMBER OF DETERMINATIONS	INDICATOR	METHOD	DEVIATIONS			
			Average	Mean	Maximum	Minimum
7	Brom phenol blue	Comparator	−0.05	0.16±	−0.38	−0.01
6	Methyl red	Comparator	+0.10	0.11±	+0.28	0.00
4		Dilution	+0.08	0.08+	+0.18	0.00
4	Propyl red	Comparator	+0.08	0.08+	+0.18	0.00
2		Dilution	±0.00	0.00	0.00	0.00
10	Brom cresol purple	Comparator	−0.01	0.04±	±0.07	0.00
5		Dilution	−0.03	0.05±	−0.14	−0.01
14	Brom thymol blue	Comparator	−0.10	0.15±	−0.25	+0.03
5		Dilution	−0.10	0.12±	−0.26	+0.01
12	Phenol red	Comparator	−0.04	0.04±	±0.07	−0.01
8		Dilution	−0.06	0.06−	−0.12	−0.02
6	Cresol red	Comparator	−0.03	0.03−	−0.07	−0.01
3		Dilution	−0.06	0.06−	−0.11	−0.02
3	α-napthol phthalein	Comparator	−0.06	0.06−	−0.12	−0.02
5	Thymol blue	Comparator	−0.04	0.09±	+0.14	−0.01
3		Dilution	−0.01	0.03±	−0.06	−0.01
3	Phenol phthalein	Comparator	+0.03	0.07±	+0.14	−0.01
3	O-cresol phthalein	Comparator	+0.03	0.07±	+0.14	−0.01

molds, and acidophilic bacteria as well as for special purposes such as the study of the gastric contents.

Brom phenol blue has given considerable trouble because almost all the solutions tested with it have been turbid. This turbidity has introduced the error of dichromatic indicator solu-

TABLE 6

Deviations of colorimetric from electrometric determinations

NUMBER OF DETERMINATIONS	METHOD	DEVIATIONS IN P_H			
		Average	Mean	Maximum	Minimum
Methyl red					
Turbid cultures of *B. coli* in 1 per cent Witte peptone, 0.5 per cent K_2HPO_4+1 per cent various sugars					
36	Comparator	+0.03	0.04±	+0.13	±0.01
34	Dilution	+0.08	0.08±	+0.18	±0.01
Turbid cultures of *B. coli* in 0.5 per cent Witte peptone, 0.5 per cent K_2HPO_4, 1 per cent to 3 per cent various sugars					
6	Comparator	+0.05	0.05+	+0.08	+0.01
11	Dilution	+0.10	0.10+	+0.28	+0.03

TABLE 7

Colorimetric and electrometric determination of the P_H *of urines. Comparator used in all cases and screen with brom cresol purple in most cases*

INDICATOR	P_H COLORIMETRIC	P_H ELECTROMETRIC
Methyl red	5.5	5.54
	5.3	5.38
	5.5	5.55
	5.3	5.33
Propyl red	5.7	5.77
	5.6	5.62
	6.0	6.01
Bromcre sol purple	6.4	6.39
	5.9	5.88
	6.5	6.67
	6.1	6.01
	6.0	6.04
	6.4	6.36
	5.7	5.62
	5.7	5.77
	5.5	5.54
Brom thymol blue	6.8	6.80
	6.5	6.67
	6.5	6.43
	6.4	6.38
Phenol red	6.8	6.80

tions which has been discussed in a previous section. Table 9, showing determinations with brom phenol blue does not, unfortunately, show any determinations made with the screened electric light. Some of these determinations are included in the summarized results of tables 4 and 5. A few measurements made with the mercury arc are shown in table 10.

TABLE 8

Comparison of P_H determinations made with thymol blue (acid range) and with hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent K_2HPO_4	Moderate color	2.4 2.0	2.4	2.37 1.97
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color		1.9	1.77
0.2 per cent $NH_4H_2PO_4$, 0.02 per cent KCl, 0.02 per cent $MgSO_4$, 5 per cent sucrose, *Aspergillus* culture	Clear		1.8	1.76
0.1 per cent $(NH_4)_2HPO_4$, 0.02 per cent KCl, 0.01 per cent $MgSO_4$, 5 per cent sucrose, *Aspergillus* culture	Clear		1.9	1.86
Whey	Very turbid		2.7 1.9	2.56 1.64
"White vinegar"	Clear		2.3	2.36

Methyl red has given some puzzling results. Table 6 shows that it may be depended upon in the study of colon cultures on the media indicated and in the range of $P_H = 4.5 - 5.5$. On the other hand, methyl red has given more or less consistently high P_H values for various Dunham's solutions (table 4) and in many other cases (table 11). Superficially this appears to be more of a so-called "salt error" than a "protein error." Some of this inaccuracy with methyl red is doubtless due to the turbidity of media acidified to such a range of P_H.

TABLE 9

Comparison of P_H determinations made with brom phenol blue and with the hydrogen electrode. (Determinations made in daylight)

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
0.2 per cent $NH_4H_2PO_4$, 0.02 per cent KCl, 0.02 per cent $MgSO_4$, 5 per cent sucrose....	Clear		4.3	4.3
1 per cent peptone, 1 per cent dried yeast..................	Moderate color, turbid	{4.0, 3.9, 3.8		4.13, 3.69, 3.68
1 per cent peptone, 1 per cent dried yeast, 1 per cent K_2HPO_4....................	Moderate color, turbid	4.2		4.14
1 per cent peptone, 0.3 per cent Liebig's extract.............	Moderate color, turbid	{4.1, 3.9		3.95, 3.41
Beef infusion, 1 per cent peptone, 0.5 per cent K_2HPO_4...	Moderate color, turbid	{4.3, 4.1, 3.8		4.59, 4.13, 3.77
10 per cent gelatine, 0.3 per cent Liebig's extract.........	Moderate color, turbid	3.9		3.71
1 per cent peptone, 0.5 per cent K_2HPO_4 (culture + HCl).........................	Very turbid		4.1 4.1	4.55
Cow feces extract (unfiltered)..	Extremely colored and turbid		4.3	4.0
Silage juice....................	Green and very turbid		{4.0, 4.0	3.91, 3.82
2 per cent egg white, pepsin digest.......................	Turbid		3.9	3.59
Cider vinegar.................	Moderate color, not turbid		3.1	3.21
Apple juice....................	Moderate color, not turbid		3.8	3.76
Prune juice....................	Very dark, not turbid		4.2	4.12

Propyl red on the whole has given somewhat better results than methyl red in spite of the fact that it is chemically so similar and in spite of the fact that propyl red is comparatively so much less soluble that it precipitates on standing in the comparison solutions. We have used propyl red chiefly to cover a narrow range of P_H between those zones covered by methyl red and brom thymol blue, a zone about $P_H = 6.0$. Recently we have discovered that this same zone may be covered much more satisfactorily by the next indicator we shall discuss.

TABLE 10

Comparison of P_H determinations made with brom phenol blue and the hydrogen electrode. (Determinations in mercury are light)

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.3 per cent Liebig's extract	Moderate color, turbid	4.3	4.4	4.45
1 per cent peptone, 1 per cent dried yeast	Moderate color, turbid	4.0	4.0	3.98
		3.8		3.72
		3.7		3.65
		3.9		3.80
		3.9		3.84
Liver infusion, 1 per cent peptone	Extremely dark, and turbid	3.7	3.8	3.70
1 per cent peptone, 1 per cent dried yeast, 1 per cent glucose, culture	Very turbid	4.0	4.0	3.98

Brom cresol purple exhibits the dichromatism discussed in a previous section and may produce the same confusion which has rendered brom phenol blue so hard to deal with. This indicator on the other hand lends itself beautifully to determinations made in the screened electric light. So used, its indications in Dunham's solutions (table 4), beef infusion media (table 5), urine (table 7) and in several other cases (table 13) are very satisfactory. This is important, for the range of P_H which may be studied with this indicator is one of great bacteriological importance. The brilliant red color of brom cresol purple in the screened

TABLE 11

Comparison of P_H determinations made with methyl red and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl	Turbid	4.8	4.7	4.49
1 per cent yeast, 1 per cent peptone	Turbid	4.8	4.8	4.55
			5.3	5.32
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent sugar (cultures)	Very turbid	5.1	4.9	4.94
			5.2	5.18
			5.2	5.15
Beef infusion, 1 per cent peptone, 0.5 per cent K_2HPO_4	Very turbid	5.5	5.4	5.25
Veal infusion, 1 per cent peptone	Very turbid	4.9		4.59
Liver infusion, 1 per cent peptone	Very dark, 5–10 dilution	5.4	5.2	5.31
			5.3	5.31
		4.8		4.67
			4.8	
Beer wort	Very dark	5.1	4.9	4.91
Carrot juice	Moderate color	5.5	5.4	5.21
Cucumber juice			5.1	5.08
Apple juice	Dark color		5.1	5.02
String bean juice	Moderate color	5.5	5.3	5.23
Prune juice	Dark color	4.8	4.5	4.41
		5.1	4.7	4.80
		5.3	5.1	5.08
Banana juice	Extremely turbid, diluted 2–10		4.7	4.62
Whey	Very turbid, diluted 2–10		5.0	4.69
Cow feces extract (unfiltered)	Extremely turbid		4.3	4.1
2 per cent egg white partial pepsin digest	Very turbid		5.0	4.9
2 per cent egg white full pepsin digest	Turbid		4.7	4.49

light has an advantage over the blues and greens of the indicator next in order, and should, when possible, be used in preference to it. The screened light is chiefly useful in the more acid region.

TABLE 12

Comparison of P_H determinations made with propyl red and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.5 per cent K_2HPO_4, 3 per cent various sugars (cultures).....	Very turbid	6.2	6.0	6.07
		5.6	5.5	5.51
		5.9	5.8	5.81
			5.8	5.59
		6.0	5.8	5.84
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl..................	Moderate color	6.1	6.0	5.91
1 per cent yeast, 1 per cent peptone.....................	Moderate color	5.3		5.32
		5.7		5.60
1 per cent yeast, 1 per cent peptone, 1 per cent K_2HPO_4..	Moderate color	6.3		6.12
1 per cent yeast, 0.3 per cent Liebig's extract.............	Moderate color	5.9		5.80
Beef infusion, 1 per cent peptone, 0.5 per cent K_2HPO_4....	Moderate color	6.2		5.97
		5.5		5.25
Beer wort.....................	Very dark	6.1	6.0	5.92
10 per cent gelatine, 0.5 per cent Liebig's extract.........	Moderate color		6.0	6.04
Hay infusion..................	Moderate color	5.9	5.7	5.81
Irish potato juice..............	Black	6.1	6.2	6.06
Apple juice....................	Dark		5.7	5.65
Sweet potato juice............	Dark	5.8	5.7	5.80

Brom thymol blue. In the conduct of this indicator, whose solutions exhibit no appreciable dichromatism, may be discerned some evidence that a blue indicator is not particularly well adapted to colorimetric determinations with colored solutions. The data in table 14 indicate that while very good determinations have been made with this indicator, the results are somewhat uncertain. This has been especially noted in the

study of beef infusion media (table 5). There is no difficulty in obtaining good approximate results with this indicator, but its color changes, especially in the greens, are deceptive and must be judged with caution if accurate data are desired. As already suggested the absorption bands of colored media themselves may

TABLE 13

Comparison of P_H determinations with brom cresol purple and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 1 per cent yeast	Moderate color	6.7		6.73
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent K_2HPO_4	Moderate color	6.5	6.5	6.43
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent sugar	Turbid cultures		5.6	5.63
		6.4	6.5	6.58
		6.4	6.5	6.61
Cow feces extract	Unfiltered, extremely turbid		6.8	6.9
			6.8	6.7
			6.8	6.7
Hay infusion	Moderately dark		5.9	5.81
			5.9	5.99
Liver infusion, 1 per cent peptone	Very dark, 5–10 dilution, 2–10 dilution		6.2	6.19
			6.2	
Veal infusion, 1 per cent peptone	Moderate color		6.3	6.21
			5.3	5.26
10 per cent gelatine, 0.5 per cent Liebig's extract	Moderate color	6.0	6.0	6.04

cause confusion in the judgment of a blue indicator even when the compensation method is used. At present we believe that brom cresol purple and phenol red may be used in sufficiently extended ranges so as to make unnecessary any extensive use of brom thymol blue.

Phenol red, which we propose as an abbreviated name for phenol sulfon phthalein, we have found to be very reliable. In

TABLE 14

Comparison of P_H determinations made with brom thymol blue and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC Dilution P_H	COLORIMETRIC Comparator P_H	ELECTROMETRIC P_H
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent glycerine	Turbid cultures	6.7	6.6	6.74
		6.7	6.5	6.69
		6.3	6.2	6.28
		6.1	6.0	6.09
		6.5	6.3	6.53
		6.4	6.3	6.27
		6.3	6.2	6.32
1 per cent yeast	Moderate color	6.3		6.80
1 per cent yeast, 1 per cent peptone	Moderate color	6.6		6.77
			6.5	6.73
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl, 0.1 per cent K_2HPO_4	Moderate color	6.7	6.7	6.63
			6.2	6.43
			6.9	7.05
1 per cent peptone, 1 per cent yeast, 1 per cent K_2HPO_4	Moderate color	7.2		7.15
		6.9		6.91
		6.7		6.68
		6.5	6.4	6.42
		6.2		6.12
Beef infusion (double strength), 2 per cent peptone	Dark color		6.8	6.14
		6.0	6.0	6.00
			6.0	6.39
			6.3	6.62
		7.0	6.9	7.07
Liver infusion, 1 per cent peptone	Very dark, 5–10 dilution		6.7	6.89
Beer wort	Very dark	6.9	6.7	6.84
Hay infusion	Moderate color	6.9	6.9	6.97
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color		6.0	6.04
Vermont maple sirup	Very dark and thick	6.8	6.9	6.75
Extract cow feces (unfiltered)	Extremely turbid and colored		7.1	7.1
			6.8	6.9
			6.6	6.7
			6.8	6.6
			6.6	6.7

TABLE 14—*Continued*

SOLUTION	REMARKS	COLORIMETRIC Dilution P_H	COLORIMETRIC Comparator P_H	ELECTROMETRIC P_H
Whey	Very turbid, 2-10 dilution		6.4 6.4	6.55 6.44
2 per cent egg white (partial pepsin digest)	Turbid		6.8	7.1
Carrot juice	Moderate color	6.6	6.5	6.43
String bean juice	Moderate color	6.9	7.0	7.15
Prune juice	Dark color	6.6	6.6	6.46
Sweet potato juice	Moderate color	6.7	6.6	6.80
Beet juice	Very dark	6.2	6.2	6.07

very few instances has it given results which could be called inaccurate. The data found in tables 4, 5, 7, and 15 need no detailed discussion. This indicator is invaluable because it is useful in the region of true neutrality. Although its praise has been sung by others, we believe that the data we here present are the first which show in any extensive manner the agreement between colorimetric determinations made with this indicator and electrometric determinations.

Cresol red. What has been said of phenol red appears to hold true for cresol red, although we have not studied it so extensively. It is very important to note that cresol red extends the range which may be accurately studied colorimetrically appreciably further into the alkaline region than phenol red, and that its color changes, from clear yellow to brilliant red, are preferable to those of the previously used α-naptholphthalein whose intermediate colors are rather muddy. Data obtained with cresol red will be found in tables 4, 5, and 16.

α-naptholphthalein, which we studied before we realized the usefulness of cresol red in the same region of P_H, has proved reliable but not so satisfactory as cresol red. Several determinations with α-naptholphthalein will be found in tables 4, 5, and 7.

Thymol blue (alkaline range) is one of those indicators with which we again encounter difficulties that appear to attend the use of a yellow-blue color change. On the whole, however, thymol blue has proved more satisfactory than its brom deriva-

TABLE 15

Comparison of P_H determinations made with phenol red and hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC Dilution P_H	COLORIMETRIC Comparator P_H	ELECTROMETRIC P_H
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent glucose	Moderate color	7.1	7.0	6.95
1 per cent peptone, 1 per cent K_2HPO_4, 1 per cent yeast	Moderate color	7.5		7.58
		7.2		7.15
		6.9		6.91
		7.7		7.68
1 per cent peptone, 1 per cent yeast	Moderate color	7.4		7.45
		8.1		8.24
			6.7	6.73
1 per cent peptone, 0.3 per cent Liebig's extract	Moderate color	7.1		7.18
		7.4		7.57
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent glycerine	Very turbid cultures	7.2		7.20
		7.6		7.62
		7.2	7.2	7.18
		7.3	7.3	7.22
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent sugars	Very turbid cultures		7.1	7.10
			7.7	7.77
			7.7	7.78
			7.7	7.70
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent sugars	Moderate color	7.1	7.1	7.10
			7.6	7.65
			7.7	7.67
			7.4	7.45
			7.0	7.07
			7.5	7.51
1 per cent peptone, 0.5 per cent Liebig's extract, 0.5 per cent K_2HPO_4	Moderate color	7.4		7.39
		8.3		8.30
		6.9		7.05
McConkey's bile salt medium	Moderate color	7.3	7.3	7.45
			8.4	8.43
Beef infusion, 1 per cent peptone, 0.5 per cent NaCl, 1 per cent glucose (overheated)	Deep color		7.0	6.83
			7.0	6.72

TABLE 15—Continued

SOLUTION	REMARKS	COLORIMETRIC Dilution P_H	COLORIMETRIC Comparator P_H	ELECTRO-METRIC P_H
0.3 per cent Liebig's extract, 1 per cent peptone, 0.5 per cent NaCl, 0.1 per cent K_2HPO_4	Moderate color	7.1 7.0	7.1 7.0	7.06 6.99
Beef infusion, 1 per cent peptone, 0.5 per cent K_2HPO_4	Moderate color	7.1		7.17
Liver infusion, 1 per cent peptone	Very dark, dilution 5–10		6.9 7.3 8.1	6.89 7.32 8.13
Veal infusion, 1 per cent peptone	Moderate color	7.0 7.7	7.0 7.5	6.98 7.66
10 per cent gelatine, 0.5 per cent Liebig's extract	Moderate color	7.3 7.5	7.3 7.4	7.41 7.58
Dorset's egg medium before coagulation	See text	7.2-7.6		7.4
Hay infusion	Moderate color	6.9	6.9 8.1	6.97 8.13
Beer wort	Very dark		7.8	7.79
Cow urine	Moderate color		7.4	7.4
Cow feces extract (unfiltered)	Extremely turbid		7.3	7.1
Sweet potato juice	Moderate color	7.4	7.4	7.38
Carrot juice	Moderate color	7.8	8.1	7.85
White potato juice	Black!	7.7	7.9	7.71
String bean juice	Moderate color	7.7	8.1	8.08
Prune juice	Moderate color	7.7	7.8	7.80
Beet juice	Black!	7.7		7.92
0.1 per cent $(NH_4)H_2PO_4$, 0.02 per cent KCl, 0.01 per cent $MgSO_4$, 5 per cent sucrose; after sterilization	Clear		7.05	7.08

tive, brom thymol blue, and we place as much confidence in it as we do in phenolphthalein which covers most of the same P_H zone. Determinations with thymol blue are given in tables 4, 5, and 18. We may call particular attention to a point frequently observed. When making determinations with thymol blue and

simultaneously with phenolphthalein or o-cresol phthalein, which are one-color indicators, we have found, when the solution under examination is colored, that the two-color indicator, thymol blue, is preferable. The approximate P_H, at least, may be discerned with much greater ease than when the one-color indicators are used. This has appealed to us forcibly.

TABLE 16

Comparison of P_H *determinations made with cresol red and with the hydrogen electrode*

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent K_2HPO_4	Moderate color	7.4	7.4	7.39
		8.3	8.3	8.30
1 per cent peptone, 0.5 per cent K_2HPO_4 (cultures)	Very turbid		7.7	7.77
		7.7	7.7	7.78
		7.7	7.6	7.70
		7.7	7.7	7.70
Veal infusion, 1 per cent peptone	Moderate color, very turbid	8.4	8.6	8.49
			7.0	6.98
			7.7	7.66
Liver infusion, 1 per cent peptone	Very dark, turbid	7.3		7.32
		8.1	8.1	8.13
Hay infusion	Moderate color	8.0	8.1	8.13
		8.2	8.3	8.40
McConkey's bile salt medium	Moderate color		7.3	7.45
		8.3	8.4	8.43
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color	7.4	7.4	7.58
2 per cent egg white, partial tryptic digest			8.5	8.4

Ortho cresol phthalein is useful as a supplement to thymol blue. Determinations made with it will be found in tables 5 and 19.

Phenolphthalein in some dozen tests made with it gave values close to those indicated by ortho cresol phthalein but with the advantage in favor of the cresol compound. We have aban-

doned phenolphthalein almost entirely for P_H determinations and whenever there has been occasion to use it, whether in determinations of P_H or in titrations, we have preferred its homologue, the more brilliant ortho cresol phthalein.

A few particular observations deserve notice. Determinations with whey taken from a cheese vat, with banana juice, cow feces, silage juice, and Dorset's egg medium, are examples of

TABLE 17

Comparison of P_H determinations made with α-napthol phthalein and with the hydrogen electrode

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC
		Dilution P_H	Comparator P_H	P_H
1 per cent dried yeast, 1 per cent peptone	Moderate color	8.1		8.24
1 per cent dried yeast, 1 per cent peptone, 1 per cent K_2HPO_4	Moderate color	8.4		8.44
		7.5		7.48
		7.7		7.68
		7.5		7.58
		8.0		7.99
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl	Moderate color	8.1	8.2	8.08
1 per cent peptone, 0.3 per cent Liebig's extract	Moderate color	8.1		8.47
Beer wort	Very dark	7.6–7.7		7.79
String bean juice	Dark		8.0	8.08
Prune juice	Dark		8.0 8.1	8.14
			8.1 8.1	8.24
Irish potato juice	Black		8.4 8.4	8.57

solutions which have been studied which were so turbid that any colorimetric determination appeared hopeless. By the use of dilution, or a comparator, or both, and by the use of the brilliant indicators we have described, reasonably good determinations were made with these solutions. Dorset's egg medium we could only place between 7.2 and 7.6 (see table 15). Accurate electrometric determinations with this medium are extremely difficult. Two determinations with freshly platinized electrodes gave 7.38 and 7.41, and the average of five deter-

minations with old and new electrodes was 7.36. If the rounded value 7.4 is taken for the electrometric determination the colorimetric limits 7.2 to 7.6 which we found are in agreement to a surprising degree, when the dense turbidity and color of this medium is considered.

TABLE 18

Comparison of P_H *determinations made with thymol blue (alkaline range) and the hydrogen electrode*

SOLUTION	REMARKS	COLORIMETRIC Dilution P_H	COLORIMETRIC Comparator P_H	ELECTROMETRIC P_H
1 per cent peptone, 1 per cent yeast, 1 per cent K_2HPO_4	Moderate color	8.9 8.4		8.91 8.44
1 per cent peptone, 0.3 per cent Liebig's extract, 0.5 per cent NaCl	Moderate color	9.4	9.5	9.60
1 per cent peptone, 0.5 per cent K_2HPO_4, 1 per cent glucose (culture)	Extremely dark and turbid	8.5		8.54
10 per cent gelatine, 0.3 per cent Liebig's extract	Moderate color	8.4	8.4	8.60
Hay infusion	Dark		9.2	9.40
Veal infusion, 1 per cent peptone	Moderate color		9.2	9.24
2 per cent egg albumin, partial tryptic digest			8.5	8.4
Irish potato juice	Black		8.9 9.0 9.4 9.4	8.97 9.44
Carrot juice	Moderate color		8.6 8.9 8.9 9.2	8.81 9.27
Beet juice	Very dark		8.4 8.7 8.8	8.57 8.75
Sweet potato juice	Moderate color		8.7 8.8	8.75
Prune juice	Very dark		9.5	9.44

Much less difficult were determinations made with such solutions as oxidized potato extract, liver infusion, deep red overheated sugar bouillons and green silage juice, the coloration of which made these solutions look almost black when in bulk. In fact, we have experienced much less difficulty with color than with turbidity.

We feel certain that had we given more attention to clear and colorless solutions the colorimetric and electrometric comparisons would have shown better agreement in many cases. In other cases we might have been able to show with greater clarity consistent discrepancies such as have appeared in tests of Dunham's solutions with methyl red. We believe, however, that a systematic investigation of "protein" and "salt" errors had

TABLE 19

Comparison of P_H *determinations made with O-cresol phthalein and with hydrogen electrode*

SOLUTION	REMARKS	COLORIMETRIC		ELECTROMETRIC P_H
		Dilution P_H	Comparator P_H	
Dunham's solution	Moderate color		8.6 9.5	8.65 9.40
1 per cent dried yeast	Moderate color		8.8	8.93
1 per cent dried yeast, 1 per cent peptone	Moderate color		8.8 9.1	8.92 9.27
1 per cent dried yeast, 1 per cent peptone, 1 per cent K_2HPO_4	Moderate color		8.3 8.9	8.44 8.91
Beer wort	Very dark	8.4		8.55
Prune juice	Dark		9.3	9.44
Sweet potato juice	Moderate color		8.7	8.75
Beet juice	Black	8.4		8.57
String bean juice	Moderate color		8.5	8.63
Hay infusion	Moderate color		9.2	9.4
10 per cent gelatine, 1 per cent peptone			8.4	8.60

best be left to more intensive investigation of particular solutions. Our present purpose has been to subject the indicators and the simple crude methods which we have described to as severe trials as could reasonably be imposed, and it may again be emphasized that we have *not selected* solutions which are particularly well adapted to colorimetric determinations, we have not adjusted the salt content of the solutions, we have not filtered off any precipitates which were found or produced by the

addition of acid or alkali, and we have sought only to attain such accuracy as could be attained in less time than that required for the electrometric determinations. The results in certain instances may be regarded as unsatisfactory by those who seek to make the colorimetric method precise, but in our opinion they are, with few exceptions, quite close enough for all bacteriological work which does not require the precision of electrometric determinations.

SECTION XV. ADJUSTMENT OF CULTURE MEDIA

The adjustment of the P_H of culture media is one of the most obvious applications of the colorimetric method as was suggested by one of us in a former paper (Clark (1915 e)). It has taken us some time to complete the investigations which were promised in this former paper, and, in the meantime, Hurwitz, Meyer and Ostenberg (1915, 1916) have to some extent anticipated our treatment of this particular subject.[19] Attention, however, may be called to the fact that these authors did not make comparisons between colorimetric and electrometric measurements upon which judgment of the accuracy of colorimetric measurements should at present be based.

The procedures suggested by Hurwitz, Meyer and Ostenberg, it seems to us, have been unnecessarily complicated. In the first place they appear to have avoided by all possible means dilution of the solutions in testing for P_H, not realizing how inappreciable is the change in P_H on moderate dilution of the solutions studied. They have suggested the use of Standardized

[19] Anthony and Ekroth (1916), discussing the adjustment of culture media in a recent paper, have made several statements concerning the theory involved with which we must disagree. We are not aware that the hydrogen electrode has ever been used in *conductivity* measurements of hydrogen ion concentration. Points of inflexion in titration curves certainly do not indicate the true neutral point, nor does the nearness of an indicator's "point" of change to this point of inflexion determine its suitability for a particular medium. We regret that the interesting data presented in this paper by Anthony and Ekroth are discussed in so many details from points of view with which ours are at variance, and that some of the work of this and other laboratories has been erroneously interpreted.

N/20 and N/1 alkali, which is of course necessary in adjustments to Fuller's scale; they have proposed a solution which cannot be accepted without more investigation than they record, namely the indicator mixed with the alkali solution used in titrating and they have burdened the subject with what we believe to be too elaborate and to some extent inaccurate stoichometrical relations. It may also be noted that these authors restricted their attention to adjustments which could be made with phenol sulfon phthalein (phenol red). It is undoubtedly true that the requirements of many bacteria, especially those which live in the blood stream, are met when media are adjusted to the blood reaction or to neutrality. But such a consideration has little weight with the plant pathologist or with one who must study the organisms of cheese, silage, wines, beers, soils, sewage, the acid loving yeasts and molds, the alkali enduring organisms or the metabolism of organisms under different environments. We consider it unwise therefore to confine adjustments to any particular range of P_H and we shall refrain from suggesting any "standard" P_H whatsoever, believing that this is a subject which should be left to separate researches and that the adoption of a standard in any particular line of investigation should be based upon experimental data rather than upon general considerations. We may however again emphasize the wide range of usefulness of the set of indicators we are describing.

The procedure we recommend may be illustrated as follows.

Having the colorimetric equipment described in section XII there is needed in addition a *roughly* standardized N/I NaOH solution relatively free from carbonate and a more dilute solution made by an *exact* 1–10 dilution of the first. This may be called the N/10 NaOH solution.

A standard beef infusion medium was to be given an initial reaction of P_H 7.4. After the original infusion had been properly diluted, heated and filtered, one per cent peptone was added. The mixture was kept warm for fifteen to twenty minutes with occasional stirring and then filtered. When cooled under a tap to room temperature the solution was made up to a known volume, less than the final volumes, and the reaction was adjusted

roughly after titrating 5 cc. with the N/10 NaOH solution in the presence of phenol red until it was judged that P_H 7.4 was reached. There were required for this 0.85 cc. N/10 NaOH. Hence to one litre of the solution were added 17 cc. of the N/1 NaOH solution. The medium was now ready for the *preliminary heating, at or near the final reaction.* Heating for fifteen minutes in the autoclave at 15 pounds pressure brought down a precipitate which was filtered out. The reaction as then determined colorimetrically (comparator) was somewhat more than 7.3. For most purposes this would have been sufficiently close to the value desired, but an attempt was made to adjust the reaction more precisely.

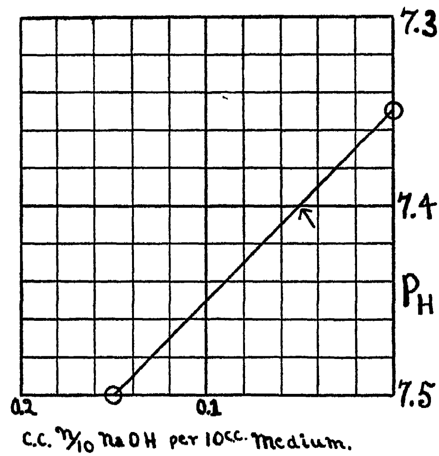

FIG. 8. PORTION OF TITRATION CURVE USED IN ADJUSTMENT OF A BEEF INFUSION MEDIUM

For a test 10 cc. were taken and readings were made with the aid of a comparator. On the addition of 0.15 cc. N/10 NaOH P_H = 7.5 was reached. This and the initial value, which was judged to be 7.35, were plotted as in figure 8. Interpolating, it was found that 7.4 would have been reached on the addition of 0.05 cc. N/10 NaOH to the 10 cc. of medium. Hence there were added to a litre of the solution 0.5 cc. N/1 NaOH. A colorimetric check showed that the desired reaction of 7.4 P_H had been

given to the medium. An electrometric determination however showed that the true reaction was $P_H = 7.47$.[20] The solution was now contaminated so it was resterilized after its volume had been brought to the desired final point. After this the reaction found colorimetrically was 7.3 and electrometrically 7.36.

This procedure may serve to illustrate *in outline* what we may suggest as a provisional method. It will perhaps need modification with special media, and in special cases. Hand in hand with the adjustment of the reaction of media must go a consideration of the substances precipitated by change in P_H, a consideration of the manipulation which is permissible, a consideration of the amount of reheating which is permissible, and a consideration of the change in P_H during sterilization. These constitute a few items of a very large subject which, we cannot treat in this paper in any adequate manner. Some of the diffi-

[20] These are about the maximum errors which we have observed with this indicator in beef infusions. We quote this particular example, however, to indicate that great accuracy cannot always be assured. Although the point attained before sterilization was 7.4, exactly that desired, when the colorimetric method alone was used, as in the studies of Hurwitz Meyer and Ostenberg, the error of 0.07 P_H was discovered when the electrometric measurement was considered as the true value.

It may be illuminating to those who are more familiar with "percentage titratable acidities" to note that in this particular solution this error amounts to about 0.07 per cent in "titratable acidity." In less strongly buffered solutions a like error in P_H would correspond to a much smaller error in "titratable acidity." In 0.3 per cent Liebig's extract, 1 per cent Witte peptone it would amount to about 0.03 per cent in "titratable acidity."

It cannot be emphasized too strongly that the reference point in determining "titratable acidities" of culture media is in reality but a P_H point indicated by the tint of phenol phthalein, that the particular tint (or particular P_H tacitly assumed) adopted by different workers varies, that the particular tint which is sometimes assumed is that at which phenol phthalein is least sensitive, and that without a proper standard comparison solution of known P_H value the same worker can hardly avoid errors of 0.1 P_H in establishing his *reference point* for "titratable acidity." Consequently "titratable acidities" which are apparently identical may vary much more widely when translated into an accurate titration curve than will the corresponding P_H values when determined by even crude methods. *Different* media adjusted to the same degree of "titratable acidity" may vary enormously in P_H. Since the publication of the paper "The 'Reaction' of Bacteriologic Culture Media" numerous instances have come to our notice of the errors, the inadequacy, and the confusion of the titrimetric method of adjusting media.

culties involved may be partly obviated by adjustments *after* sterilization such as Deelman used (see also Hurwitz, Meyer and Ostenberg (1916)). It may be suggested, however, that in a great many cases adjustment by the most direct and simple procedure will insure a medium more constant in composition and more constant in true reaction than that obtained by complex manipulation or adjustment on the old scale. We do urge that the most simple and direct procedures be given a trial before more elaborate ones creep into "standard methods." A reason for this will be discussed in section XVIII.

For the adjustment of the common solid media the following considerations may be offered. Gelatine media may be kept liquid at moderate temperatures and adjusted like liquid media. Pure agar has practically no buffer effect in the P_H ranges usually employed. Its addition to a medium should not appreciably affect the P_H of a solution, and consequently agar media may be adjusted before the addition of the agar. (High temperatures, over 40°C. should be avoided in using indicators since we have no data for these temperatures.) What effect will ensue when gel formation takes place in gelatine or agar media we may not venture to guess. If marked changes in *apparent* P_H as shown by indicators are observed, it may be found better by empirical tests to adjust to the *apparent* P_H desired as observed in the solidified medium. What interpretation other than a purely empirical one may then be put upon the indication we do not know. We are certain however that it will be quite as reliable if taken at face value as any "titratable degree of reaction" the determination of which involves implicitly the determination of a reference P_H point by means of an indicator.

Reduced to its lowest terms the colorimetric method of adjusting the "reaction" of culture media is a return to the older simple method of adjusting to a given tint of an indicator as with litmus, but with more reliable indicators, a wider choice of indicators, a logical scale of "reaction" and a clearer conception of the ends desired in adjustment. The method contains no sources of error not inherent in the titrimetric method and it avoids many sources of error which are inherent in the titrimetric method.

SECTION XVI. CONDUCT OF INDICATORS IN ACTIVE CULTURE MEDIA

It is well known that many compounds are reduced in the presence of active growths of bacteria. Reduction of colored compounds is generally accompanied by a color change. This has no *direct* connection with the color change of indicators due to alteration of P_H. Furthermore some indicators are not only reduced but fundamentally altered or destroyed by bacterial action. Therefore the conduct of an indicator when left exposed to bacterial action may be complex.

It is not to be denied that such complex conduct may furnish valuable information, just as the complex picture of numerous growth characteristics is significant to a trained eye. Nevertheless the use of indicators as indicators of P_H should be kept distinct from their other uses; and, when used as indicators of P_H, they should be introduced only at the time of the test unless used to indicate roughly the reaction. We emphasize this distinction because some have imagined that in the colorimetric measurements we are discussing, the indicators are made a part of the medium.

We have confirmed with cultures of *B. coli* Fred's (1912) observation with denitrifying organisms, that methyl red may be destroyed by bacterial action. Indeed this indicator should not be allowed to stand for more than a few minutes in active cultures. The sulfon phthalein indicators are of a very much more resistant nature. Some preliminary tests indicate that these indicators may be used to advantage in replacing other indicators which are now used in making indicator media.

We may emphasize particularly the promising value of brom cresol purple to replace the inconstant and often impure litmus or azolitmin widely used for "litmus milk." It imparts to milk a bluish grey-green color which changes on sterilization of the milk only to the extent that might be expected from the consequent change in P_H. As acid fermentations take place the color becomes yellow in easily distinguished gradations, and when alkali formation occurs a distinct blue color is imparted to the milk.

SECTION XVII. THE REACTION OF IMPORTANT SOLUTIONS

When attention is transferred from the analytically determined acid or alkali content or the so called titratable acid or alkali of solutions to the hydrogen ion concentration, some confusion is inevitable unless one has some idea of the position on the P_H scale of familiar solutions. In figure 9 are shown some of these relations. The figure is supplemented by tables 20 to 24 in which are given compilations of the P_H values of body fluids, fruit and plant juices, miscellaneous solutions and some optimal agglutination points and limiting reactions for bacteria. These compilations are given merely to help orient the true reactions of important and familiar solutions and for their suggestive value. No attempt has been made to cover the voluminous literature in detail or to present "weighted values."

SECTION XVIII. GENERAL DISCUSSION

It would be an injustice to the subject, if, after discussing its importance, its limitations and its advantages in bacteriology, we failed to indicate how the true field of usefulness of the colorimetric method is revealed by a consideration of certain broad aspects of the influence of hydrogen ion concentration.

Without fear of contradiction we may state that the hydrogen ion concentration influences the condition in solution of every substance with acidic or basic properties—native proteins and their products of hydrolysis, amines and amids, carboxyl, sulfonic and phenolic compounds, even alcoholic compounds, as well as very many of the inorganic compounds. Largely as a consequence of this the hydrogen ion concentration of a solution has a part in determining effective solubilities and the dispersion of colloids, in determining tautomeric equilibria, and in one way or another in governing the activity of catalists such as the hydrolytic enzymes and the oxidases. One or another of these effects, induced directly or perhaps very indirectly by the hydrogen ion concentration of a medium, must impress bacterial life.

Such a generalization, however, would be entirely misleading if not tempered by a proper appreciation of proportion. Rarely

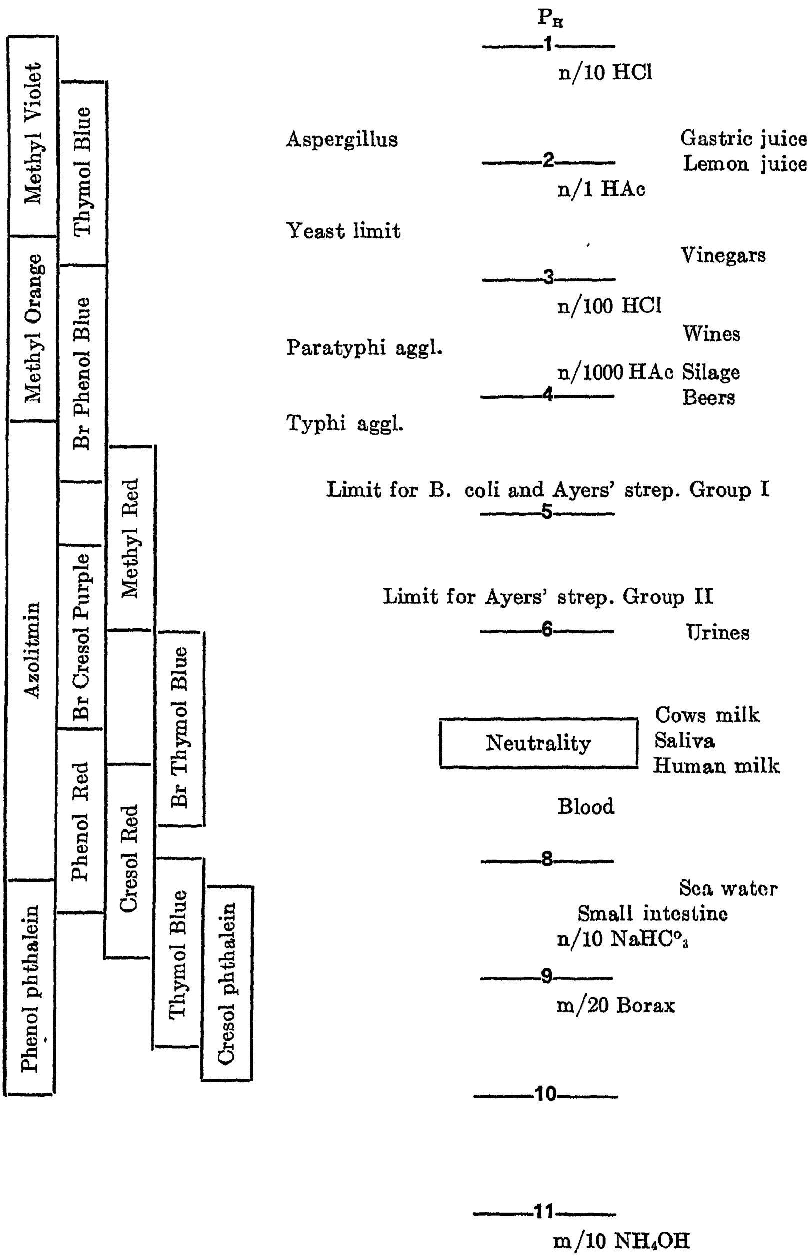

FIG. 9. THE P SCALE SHOWING THE POSITION OF IMPORTANT SOLUTIONS AND INDICATOR RANGES.

TABLE 20

True reactions of body fluids

FLUID	P_H	AUTHORITY
Blood	7.4	(See Michaelis (1914 b)
Urine	6.0[21]	(See Henderson and Palmer (1912) and Blatherwick (1914))
Saliva	6.9[22]	Michaelis and Pechstein (1914)
Gastric juice (adult)	0.9–1.6	Menton (1915)
Gastric juice (infant)	5.0	Hess (1915)
Pancreatic juice (dog)	8.3	Auerbach and Pick (1912)
Small intestinal contents	8.3	Auerback and Pick (1912)
Small intestinal contents (infant)	3.1	McClendon (1915)
Bile from liver	7.8	Okada (1915)
Bile from gall bladder	5.3–7.4	Okada (1915)
Perspiration	7.1	Foà (1906)
Perspiration	4.5[23]	Clark and Lubs
Tears	7.2	Foà (1906)
Muscle juice (fresh)	6.8	Michaelis and Kramsztyk (1914)
Muscle juice (autolyzed)	Variable	Michaelis and Kramsztyk (1914) (see Morse (1916))
Pancreas extract	5.6	Long and Fenger (1916)
Peritoneal fluid	7.4	Foà (1906)
Pericardial fluid	7.4	Foà (1906)
Aqueous humor	7.1	Foà (1906)
Vitreous humor	7.0	Foà (1906)
Cerebrospinal fluid	7.2	Foà (1906)
Cerebrospinal fluid	8.3	Hurwurtz & Tranter (1916)
Amniotic fluid	7.1	Foà (1906)
Amniotic fluid	8.1	Löb and Higuchi (1910)
Milk (human)	7.0–7.2	(See Clark (1915 b))
Milk (cow)	6.6–6.8	(See Clark (1915 b))
Milk (goat)	6.6	Foà (1906)
Milk (ass)	7.6	Foà (1906)

[21] This is a purely "general average." The reaction of urine varies considerably.

[22] The true reaction of saliva is worthy of more extensive investigation from the bacteriological point of view. The value $P_H = 6.9$, which is given by Michaelis and Pechstein, is almost exactly that of absolute neutrality. If applied at 37°C. it is more alkaline than acid, since the neutral point at 37° is about $P_H = 6.8$. Several salivas which we have tested colorimetrically were found to be 6.8–6.9, yet these were distinctly acid to both red and blue litmus paper, and would therefore be called "acid" salivas. It is not to be denied that, so far as the action upon the constituents of the teeth is concerned, the relation of this reaction to absolute neutrality is of little consequence. Rather must it be

TABLE 21

True reactions of miscellaneous solutions

SOLUTION	H	AUTHORITY
Soil extracts	4.5–8.7[24]	Gillespie (1916)
Sea water	8.0–8.4	Palitzsch (1911 a)
Mineral waters	6.5–7.0	Michaelis (1914 b)
Flour extract	6.0–6.5	Jessen-Hansen (1911)
Tan liquors	3.5–4.7	Wood Sand and Low (1911)
Vermont maple sirup	6.8	Clark and Lubs
Beers	3.9–4.7	Emslander (1914)
Wines	2.8–3.8	Paul (1914)
Cider vinegar	3.1	Clark and Lubs
"White vinegar"	2.6	Clark and Lubs
Corn silage juice	3.7[25]	Clark and Lubs
Human feces (adults')	7.1–8.8	Howe and Hawk (1912)
Human feces (infants')	6.0–7.0	(See Michaelis (1914 b))
Cow feces	6.6–7.2	Clark and Lubs
Snail lymph	9.0	Foà (1906)

related to the solubilities of the constituents of the teeth at different P_H. Nevertheless this neutral reaction of so-called "acid" salivas is a good illustration of the confusing connotations which have been attached to the term acidity.

[23] The value of $P_H = 7.1$ was obtained by Foà (1906) on perspiration whose flow was stimulated by a hot air bath and from skin which had been washed with alkali to remove the fatty acids. These fatty acids, it has been claimed, are not normal constituents of the perspiration but are excreted by the sebaceous glands. Whatever may be the value of Foà's determination for the student of the mechanisms by which the body reactions are regulated it is of little interest to the bacteriologist when compared with the importance of the reaction of the perspiration as it normally occurs. It has been very commonly held that the reaction of the perspiration is variable but generally acid. Park and Williams (1910) state "The secretions of the sebaceous glands appear to be little, if at all, bactericidal, but the perspiration, on account of its acidity, is slightly so."

Three samples which we collected from clean normal skin had a reaction of about $P_H = 4.5$. Whether this is extreme and exceptional or not remains for others to determine. We may however note that such a reaction will doubtless tend to keep dormant many bacteria even if it does not exert a distinct bactericidal action.

[24] This wide variation in the acidities of soil extracts cannot fail to indicate some wide differences in the effective acidities of the soils themselves. These observations by Dr. Gillespie, which are the most enlightening data on the mooted question of soil acidity, are of profound importance to soil bacteriology.

[25] This acidity of silage juice is considerably higher than that attained in cultures of any of the streptococci we have yet studied and is practically the P_H that some of our unpublished researches have shown the bacteria of the Bul-

TABLE 22

True reaction of fruit and plant juices

SOLUTION	P_H	AUTHORITY
Lime juice	1.7	Patten and Mains[26]
Lemon juice	2.2	Patten and Mains[26]
Cherry juice	2.5	Patten and Mains[26]
Grapefruit juice	3.0–3.3	Patten and Mains[26]
Orange juice	3.1–4.1	Patten and Mains[26]
Rhubarb juice	3.1	Patten and Mains[26]
Strawberry juice	3.4	Patten and Mains[26]
Pineapple juice	3.4–4.1	Patten and Mains[26]
Tomato juice	4.2	Patten and Mains[26]
Pear (nearly ripe)	4.2	Foà (1906)
Grape juice	4.5	Foà (1906)
"Normal plant cell sap"	5.3–5.8	Wagner (1916)
Fiscus elastica milk	5.7	Foà (1906)
Prune juice (autoclaved)	4.3	Clark and Lubs
Apple juice (autoclaved)	3.8	Clark and Lubs
Banana juice (autoclaved)	4.6	Clark and Lubs
String bean juice (autoclaved)	5.2	Clark and Lubs
Carrot juice (autoclaved)	5.2	Clark and Lubs
Cucumber juice (autoclaved)	5.1	Clark and Lubs
Beet juice (autoclaved)	6.1	Clark and Lubs
Potato juice (autoclaved)	6.1	Clark and Lubs

is it necessary, for instance, to consider the dissociation of sugars, since their acid dissociation constants are so small that these substances are generally found only in a practically molecular state in neutral or acid solutions. Likewise there are zones within which any given acidic or basic group will be found in a practically totally dissociated or practically undissociated state. Perhaps there is no more vivid way of illustrating this than by a contemplation of indicators. Above a certain zone of hydrogen ion concentration phenolphthalein solutions are colorless. Below this zone (until intense alkalinity is reached) only the colored form is observed. Within the zone the color change

garicus type to reach. This observation is of very interesting significance now that Hunter and Bushnell (1916) and Sherman (1916) have shown the dominant organisms in silage to be of the Bulgaricus type.

[26] These values were obtained by Dr. Patton and Mr. Mains of The Bureau of Chemistry. By the courtesy of Dr. Patton and Mr. Mains and of the Bureau of Chemistry we are permitted to publish these average values in advance.

with change in hydrogen ion concentration is great. The conduct of phenolphthalein, which happens to be visible because of tautomeric changes which accompany dissociation, is a prototype of the conduct of all acids. Just as we may suppress the dissociation of phenolphthalein by raising the hydrogen ion concentration of the solution, so we may suppress the dissociation of any acid. A similar illustration for the conduct of bases is obtained if we regard methyl red as a base. The general

TABLE 23

Acid agglutination optima

ORGANISM	P_H	AUTHORITY
B. paratyphi	3.7	Michaelis (1911)
B. typhi	4.4	Michaelis (1911)
Pneumococci	5.0	Gillespie (1914)
Plague bacillus	5.0	Markl (1915)

TABLE 24

Limiting reactions

ORGANISM	P_H	AUTHORITY
B. coli	5.0	Michaelis and Marcora (1912)
B. coli (low gas ratio group)	4.3–5.3	Clark (1915c)
Streptococci, Group I	4.6–4.8	Ayers (1916)
Streptococci, Group II	5.5–6.0	Ayers (1916)
Streptococcus erysipelatos	4.8	Itano (1916)
B. subtilis, acid limit	4.2	Itano (1916)
B. subtilis, alkaline limit	9.4	Itano (1916)
Yeast	2.5–2.7	Lüers (1914)
Yeast	2.3	Högglund (1915a)

relations between the strength of acids as expressed by their dissociation constants and the percentage dissociation at different hydrogen ion concentrations will be found graphically illustrated in figure 5 (Journal of Bacteriology, ii, 110).

The point we wish to emphasize is, that within certain zones of hydrogen ion concentration slight changes may have an enormous effect just as we observe them to have in the case of indicators; while in other regions no appreciable effect is observed although the hydrogen ion concentration is varied enormously.

An important instance where such a consideration is very suggestive has been noted by Michaelis (1914 b). Michaelis (1914 a) and his collaborators (e.g., Michaelis and Davidsohn (1911)) have shown that enzyme solutions conduct themselves as if the enzyme were an electrolyte. Invertase, for instance, gives an activity curve for different hydrogen ion concentrations which has the form and many of the characteristics of the dissociation residue curve of an amphoteric electrolyte. In such curves there is seldom a sharp optimum point as has sometimes been inferred, but rather an optimum *zone*. Within and beyond this zone slight changes in hydrogen ion concentration produce no great change in the activity of the enzyme, but upon the borders of the zone a small change in P_H may make a very large difference. Michaelis suggests that there may be maintained in the organs of the body hydrogen ion concentrations which lie upon the border of the zone of optimal activity of a given enzyme and that this permits the rate or the direction of enzymatic action to be controlled by slight changes in P_H.

Considerations such as these, which will become more apparent if one studies the beautiful exposition of the dissociation curves of acids, bases and amphoteric electrolytes given by Sörensen (1912) and Michaelis (1914), would lead one to believe that the methods of determining hydrogen ion concentrations should be developed to meet two general classes of problems. In the first place, when the phenomenon under investigation is connected with an electrolytic equilibrium occurring in a P_H region where the equilibrium is seriously disturbed by slight changes in P_H the method of determining P_H values should be the most reliable available. In the second case, when the equilibrium is held practically constant within a certain wide zone of hydrogen ion concentration, it will do for most purposes to determine P_H by some approximate method. Neglecting the question of availability and arguing only from the basis of scientific efficiency the electrometric method should be applied in the first case because of its greater accuracy and the colorimetric method in the second case because of its simplicity. When the nature of the process investigated is not known and we are there-

fore unable to tell *a priori* which method on the above basis should be used, then a preliminary survey may be made with the colorimetric method. This it seems to us furnishes a rational basis for judging the true usefulness of the two methods.

Exception will be taken to this point of view as a comprehensive basis since there are cases in which the electrometric method is inapplicable and the colorimetric method must be used in preference, or *vice versa.* Nevertheless, we believe that in general the true utility of the colorimetric method lies, first in its availability where approximate determinations are needed but where exact determinations are useless, and second in its value for reconnaisance.

In some instances the mere fact that a phenomenon will occur over a considerable range of P_H should be clearly distinguished from the significance of an optimum point. A case in point is the acid agglutination of bacteria. An analogy which is imperfect, but which may serve to illustrate this, is found in the conduct of indicators. Phenol red and cresol red are distinct compounds of the same series which we may compare for present purposes with two distinct strains of bacteria of the same species. The two indicators conduct themselves in a very similar way. To a casual observer their colors would appear very much alike and their color changes would appear to occur in the same range of P_H. If we introduce these indicators into standard buffer mixtures a red color will be observed with each at P_H 7.4, 7.6, 7.8, etc., just as acid agglutination of bacteria may be observed at, say P_H 4.8, 5.0, 5.2, etc. But the *half transformation point* of each indicator is *characteristic* and may be used to *identify* the compounds. Likewise it is the *optimum* agglutination point which is *characteristic* of bacteria or of their protein.

It may not be out of place to prolong this discussion, keeping in mind the general point of view we have presented, and to indicate more concretely whese the colorimetric method may be applied in bacteriology.

In regard to the "reaction" of culture media several facts have become apparent. In the first place, adjustments by the old titrimetric method gave widely divergent hydrogen ion con-

centrations in media of different composition and not very close agreement in different samples of the same medium. In spite of this, media so adjusted were successful in many cases. This would indicate that rough adjustment of the hydrogen ion concentration is all that is necessary. Such adjustments may be adequately controlled by the colorimetric method. If they are, we shall not again encounter situations like that which occurred when the composition of a standard official medium was changed without revision of the "standard degree of reaction," the P_H of the revised medium being at about the limit endured by the organism the medium was intended to nourish.

But although the rough adjustment of the P_H of culture media may serve in most cases, we should not overlook the fact that many organisms have been described as very sensitive to "reaction." As one of us pointed out in a former paper we do not know whether this sensitivity has been overestimated because buffer poor media were used or because of the inaccuracies and uncertainties of the titrimetric method, or whether it is a true sensitivity. For a reconnoisance of this problem the colorimetric method should prove valuable.

As media become simplified and approach closer to the ideal of synthetic composition they can be made up with greater assurance of a constant initial P_H. The establishment of particular reactions is especially easy when there are present phosphates or similar well defined substances which exert strong buffer action at certain zones of P_H to the advantage of bacterial growth as Sörenson has pointed out. Henderson has repeatedly emphasized the value of mixtures of basic and acid phosphates in regulating solutions at or near the neutral point, and Henderson and Webster (1907) have specifically recommended such a mixture for prolonging the activity of "lactic bacteria." It may be noted however that the *addition of mixtures* of the phosphates to media is not always necessary. Phosphates have relatively little buffer action at hydrogen ion concentrations lower than that of blood. Consequently, if a secondary phosphate is added to a medium which is relatively rich in base binding compounds, the secondary phosphate cannot greatly lower the hydrogen ion

concentration. On the other hand at and above neutrality the phosphate will exert a strong buffer action and tend to keep the medium near the neutral point. Consequently the addition of a proper quantity of K_2HPO_4 to an acid medium is an effective way in which approximately to adjust the medium to neutrality. The use of di ammonium phosphate is even safer because it cannot retain an excess of base, especially after heating, and as ordinarily obtained is a mixture of the mono with an excess of the diammonium phosphate.

Synthetic media present some special problems. Not a few of those which have been proposed have so little buffer effect that they are practically useless and although "synthetic" are physiologically variable because of the enormous difference in P_H which very slight impurities may produce. With the proper set of indicators this may be easily discovered. Media have also been proposed which, in the language of the druggist, contain "incompatibles." The original Czapek (1901) medium for molds contained magnesium and a phosphate, but the magnesium was kept from precipitation as magnesium phosphate by using an acid phosphate. Copyists have neglected to specify this and, in some cases, have even specified the use of a basic phosphate. The resulting P_H permits the precipitation of the magnesium. Doubtless there remains even in such a solution a sufficient quantity of magnesium to supply the minimum requirement, but since magnesium is one of the essential elements for mold growth it is better technique, at least, to keep in solution that which is supplied.

Certain similar aspects of synthetic media will be discussed more fully in a subsequent paper, but before leaving the topic we may call attention to the well known fact that the reaction of a medium has a considerable influence upon the decompositions which occur during sterilization. Pure glucose and pure "microcosmic salt" form an excellent medium for many purposes, but after sterilization such a solution may hardly be called a synthetic medium in the strict sense of the term. Likewise the more complex media suffer changes on sterilization which in some instances especially on overheating, are of serious impor-

tance. Among many items of special interest to media makers may be mentioned the formation of complexes between sugars and amino acids, treated by Maillard (1913), the decomposition of asparagine, an important constituent of many synthetic culture media, which has been studied by Ehrlich and Lange (1913), innumerable researches on the cleavages of sugars in alkaline solutions, and the reports of Lobry de Bruyn and Alberta van Ekenstein (1895–1900) on the transformations of sugars into one another in alkaline solutions. These last are of such grave importance to bacteriologists that most careful confirmation may be demanded. It may not be too speculative to suggest at this point that in some instances the baneful effect of overheating media may be due to the destruction of special compounds which in small quantities are essential to the best activity of fastidious organisms. It is well known that certain so-called "vitamines," which are essential to the health of higher organisms and without which disease and death ensue, are destroyed upon heating, especially when the reaction of the solution is slightly alkaline or even neutral. It is not at all impossible that analogous compounds necessary for the continued growth of highly specialized bacteria may be destroyed by sterilization at the reactions usually employed. The later work on "vitamines" recalls the earlier work on Wildier's (1901) "bios," a substance which he describes as "indispensable to the development of yeasts" (see also Amand (1903–04)). Devloo's (1906) description of this sounds like a modern paper on a vitamine. Of particular interest to the present discussion is the statement that it is stable in acid solutions but unstable in alkaline solutions (like the vitamines).

Now Michaelis and Rona (1906 b) have emphasized, more particularly with reference to the alleged decomposition of sugar at the temperature and alkalinity of the blood, that we should not transfer indiscriminately to the conditions of physiological solutions the conclusions of those who have worked with molecular concentrations of alkali or acid. The effective alkalinity or acidity as measured by the hydrogen ion concentration may be thousands of times greater in the one case than in the other.

Therefore much of the data on the decompositions which occur in bacteriological culture media and the influence thereon of "reaction" had best be studied with at least such control as the indicator method will afford.

When a correlation is observed between P_H and some effect, the mere determination of P_H alone will of course throw but little light upon the real nature of the phenomenon except in rare instances. Determination of the hydrogen ion concentration will not even distinguish whether a given effect is influenced by the hydrogen or the hydroxyl ions, nor will it always reveal whether the influence observed is direct or indirect. It is true however that even when the hydrogen ion concentration is effective through remote channels it may be very important. Therefore advantage should be taken of the comparative ease with which the concentration of hydrogen ions may be determined or controlled and their influence known or made a constant during the study of any other factor which may influence a process. From this point of view methods of determining hydrogen ion concentration take their place beside thermometers, and buffer mixtures beside thermostats. In some instances automatic regulation of a hydrogen ion concentration may be provided, to take its place beside the automatic thermo regulator. For instance, Dr. Jones of this laboratory provided a very good automatic regulation of the P_H of a medium at a desired point by keeping the medium saturated with CO_2. Since anaerobiosis was also desired the procedure in this case was one of unique beauty. For alkaline solutions a modified use of the ammonia vapor tension of ammonia solutions might do.

The influence of hydrogen ion concentrations upon solubilities has been mentioned. It extends quite naturally to many of those substances which exist in solution in aggregates which bring them within the classification of colloids. Whether by reason of its influence on dissociation or because of other phenomena the hydrogen ion concentration of a solution may influence the dispersion of colloids. Among such substances of interest to the bacteriologist are bacterial enzymes, certain toxins, and the components of bacteria and viruses which may

react as if the bacteria themselves were dispersible, and several of the membranes used for the separation of enzymes, toxins bacteria and viruses from their media. Whether certain of these enzymes toxins, etc., would have appeared in the filtrates obtained by certain workers, had the medium been given the proper reaction, it is difficult to say; but it is worth while to call attention to the remarkable observations of Maurice Holderer (1911) which have been confirmed in many details by others. He not only controlled the filterability of enzymes[27] by adjusting the reaction of their solutions with the aid of indicators, but he also obtained new enzymes from certain organisms by extractions at certain reactions. Aubel and Colin (1915) have confirmed Holderer's conclusions with some bacterial toxins.

In the study of all of these problems, in the testing of acid and alkali fermentations, in the investigation of such influences as the reaction of the medium is reported to exert on the formation of toxin by the diphtheria bacillus [see Park and Williams (1910)) and Ficker (1913)], and especially in the study of that neglected but profoundly important subject,—the relative *rates* of different bacterial enzymatic processes, the indicator method may be used with advantage, at least for reconnoissance.

There is always danger that so important a subject will be either overrated or underrated before its position has been clearly defined by extensive investigation. Let us therefore recall the view point established by Sörensen in his classic paper "Enzymstudien II." As he said, the influence of hydrogen ion concentration is analogous to that of temperature. In each case there are limits beyond which the action of enzymes practically ceases. In each case there is a broad optimal zone. In each case there is at least upon one side of this zone a region where slight changes in the condition produce great changes in the rate of action. May we not extend this analogy to bacterial activity in general, and maintain that, just as we pay attention to the temperature of the incubator, so should we pay attention

[27] Since writing the manuscript of this paper one of us (W. M. C.) has found that the passage through Berkfeld filters of the gelatine liquefying enzyme of B. proteus can be controlled by adjusting the P_H of the solution.

to the hydrogen ion concentration of the medium. For general work precise thermometry, as the physicist knows it, has no place in bacteriological technique; for general work precise hydrogen electrode measurements of P_H are unnecessary. But a consideration of the facts already established is making it evident that we must have some means of determining hydrogen ion concentration which is comparable with the use of an ordinary thermometer for determining temperature and that it is advisable to use it with the same frequency. Experience with temperature effects enables one to judge of the accuracy required in any particular case; only experience will furnish a true measure of the limitations as well as the advantages of the indicator method. Our present judgment is that certain bacteriological problems are worthy of the instrumental accuracy of the very best hydrogen electrode equipment and of the most careful and skilled technique that can be applied to that instrument, but that to discover the cases where this may be necessary as well as to deal with numerous problems where this is surely not necessary the indicator method is adequate.[28]

[28] It is perfectly obvious that the indicators and the methods which we have described in this paper with particular reference to the needs of bacteriology may prove to be useful in other fields of investigation. The excellence of some of the indicators for the determination of the P_H values of urines is shown in table 12. Dr. Gillespie (1915) has used some of the new indicators in his soil work, and Dr. Patton and Mr. Mains have used them in studies on the acidities of fruit juices. Many other special uses might be suggested.

It is perhaps less obvious that many of the considerations which have led us to abandon the titrimetric method of adjusting the reaction of culture media apply to a great many other tests. Extracts of natural products very frequently give titration curves which are similar in many respects to the curves of culture media already described. One important characteristic is the strong buffer effect exhibited in the P_H region where phenol phthalein changes. It can easily be shown that such solutions are not subject to the application of the principles which have been developed for the titration of pure solutions of strong and moderately weak acids. In the great majority of cases the titration of such solutions reduces to a method of showing mere differences in different samples without showing the analytical content of the acids or bases.

According to Trillat (1916) the ancient Romans titrated the alkalinity of natural waters with drops of red wine. Although modern standards of concentration are more exact than the wine standard used by the Romans the modern

SECTION XIX. SUMMARY

Recent developments in the study of the influence of the hydrogen ion upon various processes of physiological importance have made it evident that these studies must be extended to bacteriological problems. We have given a very brief sketch of work already accomplished in other fields and have noted some instances where the influence of hydrogen ion concentrations upon the activities of bacteria, yeasts and molds has already been demonstrated.

With due consideration for certain superiorities in the electrometric method of determining hydrogen ion concentrations we have pointed out that for general work the colorimetric method promises to be most useful.

Certain major and minor hindrances to its general application in bacteriology we have materially lessened or removed. A new series of indicators of exceptional brilliancy has been assembled from among those already described or from the several new indicators which we have synthetized for the first time. The selection is composed of the following individual compounds with which are listed the useful ranges.

choice of indicators is oftentimes no more logical than the Roman choice of the coloring matter of red wine.

The frank admission that the analytical content of acid or base in a given complex solution cannot be determined by titration to a given tint of some arbitrarily selected indicator does not necessarily destroy the empirical value of such a titration. There can be no doubt, for instance, that the titration of milk furnishes information of great value to the cheese maker or the creameryman, but it is nonsense to elaborate the simple straightforward procedure of titration so that the results even in the case of perfectly fresh milk must be stated as *percentage lactic acid!*

In the titration of the various extracts which have to be dealt with by some empirical method in food or inspection laboratories it may frequently happen that significant differences can be detected better by a simple rapid colorimetric test of the original P_H values of different samples. In other cases it may be better to titrate to some point other than the customary phenol phthalein point or rather region. In either case the brilliancy and what we now know of the reliability of the indicators we have had the pleasure of describing will, we are sure, appeal to others.

	Range P_H
Thymol blue (acid range)	1.2–2.8
Thymol blue (alkaline range)	8.0–9.6
Brom phenol blue	2.8–4.6
Methyl red	4.4–6.0
Propyl red	4.8–6.4
Brom cresol purple	5.2–6.8
Brom thymol blue	6.0–7.6
Phenol red	6.8–8.4
Cresol red	7.2–8.8
Cresol phthalein	8.2–9.8

These indicators have been tested in a wide variety of solutions such as are used for the cultivation of bacteria, and especially in colored and turbid media, and their indications compared with electrometric measurements. The correlation reveals a general agreement which may be considered satisfactory for most purposes. In certain instances discrepancies which may be attributed to "protein" or "salt" errors and in some instances discrepancies which were traced to optical effects have shown that the indicators are not always reliable for accurate measurements.

In the colorimetric determinations the Walpole compensation method for colored and turbid media has been extensively studied with the simple device of Hurwitz, Meyer and Ostenberg. The dilution method has also been elaborated. Approximate and systematized procedures have been described. Special apparatus of simple design including a special light source for use with the two dichromatic indicators have been devised.

A new set of standard comparison solutions especially designed for accuracy and ease of preparation has been described, and, as reported elsewhere, carefully studied.

Brief sketches have been given of the more important theoretical aspects of the use of indicators, and general considerations have been presented to show the proper field of usefulness of the method.

It is concluded that, with the improvements presented, the colorimetric method is available for routine as well as research purposes in bacteriology.

Applications already made have been mentioned and new uses have been suggested.

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THE USE OF A THREE PER CENT LACTOSE LITMUS AGAR PLATE FOR THE DEMONSTRATION OF B. COLI IN WATER EXAMINATION

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The use of standard lactose litmus agar plates (A. P. H. A., 1912, p. 130; A. P. H. A., 1917,) for the confirmation of the presence of *B. coli*, is complicated by many objectionable features (Frost, 1911, pp. 121, 133; Prescott and Winslow, 1915, pp. 105, 132; Rector, 1913). Organisms other than *B. coli* grow readily on this medium and are apt to spread over the surface of the plate. This spreading either prevents the growth of the colon bacilli or masks their typical reaction and often renders the fishing of colonies for further identification either difficult or impossible. Also, as a rule, in order to insure proper distribution of colonies, two or more plates must be made for each tube to be confirmed.

Streak plates made upon Endo's fuchsin-sulfite medium have been used in many laboratories to offset these difficulties. Endo's medium is made with a 3 per cent agar base (A. P. H. A., 1912, p. 133). Plates are poured and allowed to harden thoroughly and inoculation is made by streaking out a loop of the liquid medium to be confirmed, onto the surface. After a little experience in their use, good distribution of colonies on these plates may be secured, using only one plate for each tube. Endo's medium plates are very restrictive in action and this fact has been ascribed to the fuchsin and sulfite. The results obtained by the use of the medium described in this paper would however, seem to indicate that most, if not all, of the restrictive action is due to the high concentration of agar used.

In spite of the advantages of Endo's medium, lactose litmus agar plates are preferred by many water-workers (Frost, 1911, pp. 121, 133; McLaughlin, 1913, p. 12), and it is the confirmatory medium in most common use. Frost has shown that the ordinary (1 per cent agar-poured) lactose litmus agar plate and the Endo's medium plate are equally diagnostic as to confirmation of presumptive tests (Frost, 1911, pp. 118, 121). Choice should therefore be based on considerations of convenience.

In an attempt to eliminate the difficulties incident to the use of lactose litmus agar, this medium was made up using the regular 3 per cent agar "base" as made for Endo's medium, prepared with the 1 per cent peptone and 1 per cent meat extract. As used by the writer, the base was put up in 400 cc. portions, to which were added when ready for use, 16 cc. of a sterile 25 per cent lactose solution, and 12 cc. of a sterile 8 per cent litmus solution. This amount of medium was used to pour 25 to 30 plates, which were allowed to harden open to the air. Plates not immediately used were stored at ice-chest temperature, without any apparent change in appearance or results. This medium seems just as restrictive to air forms as Endo's medium. Five or six plates, made as above and subject to normal air contamination during thirty minutes of hardening, showed no growths after fourteen days storage in the refrigerator.

This method of making lactose litmus agar plates could not, of course, be used for direct plating of water or sewage samples for a "red colony" count, without much difficulty in manipulation (Frost, 1911, p. 121). In general, however, the direct red colony count has been definitely discarded in favor of the dilution method, for quantitative determinations of *B. coli*. Therefore, the only use for lactose litmus agar is as a confirmatory medium.

To check this new medium against Endo's medium, fermentation tubes showing gas were inoculated simultaneously onto the surface of plates of both media. When typical colonies appeared on both plates, the demonstration was carried no further. When the colonies on either or both media were atypical, one or more were fished from each plate. These were inoculated into lactose bouillon tubes and onto agar slants. If more than 10

per cent gas was formed within forty-eight hours and the slant showed only Gram negative non-sporeforming bacilli, the colonies were considered *B. coli.* It was found that in all cases where the tubes showed gas, this was due to Gram negative, non-sporeforming organisms.

Summary of results of comparison of 3 per cent agar lactose litmus plates with Endo's medium plates

	NUMBER OF TUBES PLATED	NUMBER SHOWING B. COLI	
		Lactose litmus agar 3%	Endo's medium
Plates typical with both	101	101[1]	101[1]
Plates atypical with both	65	5[2]	13[2]
Typical lactose litmus agar, atypical Endo	2	1[2]	1[2]
Atypical lactose litmus agar, typical Endo	24	6[2]	13[2]
Total	192	113 (59%)	128 (66.6%)

[1] Considered *B. coli* without further confirmation.

[2] Demonstrated as *B. coli* Gram-negative, non-spore-forming bacilli, giving gas from lactose.

Note must be made of the fact that the low percentages of confirmations, 59 per cent for the lactose litmus agar and 66 per cent for the Endo's, were due to the fact that only tubes originally showing less than 10 per cent of gas in twenty-four hours were used for this work. The average confirmation for all tubes, for this laboratory, for the year 1914, was about 92 per cent.

SUMMARY

Lactose litmus agar plates prepared with 3 per cent agar and inoculated by streaking onto the surface are free from many objections incident to standard lactose litmus agar plates. They are more convenient, restrict entirely any spreading forms, and give results approximately equivalent to Endo's medium plates. The latter, however, appears in the writer's estimation to be somewhat preferable, as giving slightly higher results.

The writer wishes to acknowledge the kind suggestions of Dr. W. H. Frost in the preparation of this paper.

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A SIMPLE METHOD FOR STAINING THE CAPSULES OF BACTERIA

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The possession of mucinous envelopes by certain bacteria, apart from their purely morphological interest, has a great value in the identification of such organisms and many methods have been devised for their demonstration. The number of such methods however argues against any one being completely satisfactory.

The introduction of a new technique into this already overcrowded field can be justified only if it offers distinct advantages over any or all of the older methods. The staining of capsules as a rule, particularly in inexperienced hands, is time consuming and attended by many failures, and a new method to be acceptable should as far as possible eliminate those factors which experience has shown to be the most general cause of failure.

These factors may be the uncertain or variable quality of the diluent employed, the impossibility of giving absolute directions as to the amount of heat required in fixation or in staining, or the use of fixing agents, such as strong chemicals, that in themselves tend to shrink the capsule.

The search for a method that would overcome these difficulties led to the employment of an artificial menstruum prepared from Nutrose[1] (as directed below), the abandonment of heat as a fixing agent and as an aid in staining, and the use of weak solu-

[1] Nutrose (sodium caseinate) is a proprietary food preparation made in Germany and, as under present conditions it may be unobtainable, a fairly efficient substitute for the 3 per cent Nutrose solution may be made from milk by the following procedure: Render milk as nearly fat free as possible by means of centrifugation, add 1 per cent of 2 N sodium hydrate and bring to a boil, after cooling add ether and shake. After a few minutes decant off the ether. The remaining opalescent fluid can then be employed in place of the Nutrose solution.

tions of lactic acid as precipitating agents. Thin films made from an emulsion of organisms in 3 per cent Nutrose solution are precipitated immediately by dilutions of lactic acid of a strength of 0.1 of 1 per cent causing a perfect fixation of the organisms and capsules.

Experience has shown that 0.25 to 0.5 of 1 per cent of lactic acid gives the best results, that it is perfectly feasible to introduce the dye employed directly into the fixing solution, thus simplifying the technique, and that the addition of acetic acid in a strength of 1–10,000 improves the definition.

Method of preparing the reagents employed

Solution 1. To be used as a diluent.

Three grams of Nutrose are sifted into 100 cc. of distilled water and heated to 100°C. in the Arnold sterilizer for one hour.

Add 5 cc. of 2 per cent aqueous solution of carbolic acid to serve as a preservative. Decant into test tubes and allow to settle.

Employ the supernatant fluid as the diluent.

(Since the supernatant fluid tends to become thinner by constant precipitation of the Nutrose the solution should occasionally be reboiled.)

Solution 2. Fixing and staining solution.

2 per cent aqueous solution of carbolic acid, 100 cc.
Concentrated lactic acid, 0.25–0.5 cc.
1 per cent acetic acid, 1 cc.
Saturated alcoholic solution of basic fuchsin, 1 cc.
Carbol-fuchsin (old), 1 cc.

(This solution must be kept tightly corked, and then keeps very well.)

Experience has shown that in the above solution the addition of old and fresh fuchsin as given makes a better product than an increase in the amount of either one alone.

Technique of staining

1. Employ the Nutrose solution (no. 1) as a diluent, emulsifying the bacteria in one or two loopfuls and then spreading in as thin a film as possible with the loop. The use of the edge of a slide in spreading the film, as in blood work, is not to be recommended.
2. Allow to dry in air.
3. Cover the film with the fixative and staining solution (no. 2) and allow to act for thirty to forty-five seconds.
4. Wash quickly in water, dry and examine.

These steps take about one and half minutes.

In examining the films the best pictures are usually found in the thinnest portions near the edges.

The use of fuchsin as a dye has proven to be generally satisfactory for a routine stain but any of the more diffuse basic dyes may be employed. Methyl violet added in 1 per cent of a saturated alcoholic solution gives probably the best pictures due to the failure of the Nutrose film to stain, the organisms appearing as blue black bodies surrounded by delicate violet capsules. This dye, however, tends to precipitate out of the mixture in a few days and the preparations are apt to fade.

The above method has been in use in this laboratory for three years and has been used in the class room during this period with very satisfactory results, being taught in conjunction with one of the older methods so that a comparison of results on a very large number of preparations was possible. It rapidly became the method of choice by students due to the simplicity of the technique and the very high percentage of successful results secured. The results of preparations made by this method are shown in figures 1 to 6.

Since the presence of a demonstrable capsule has such differential value, a method of this kind to be acceptable must fail to show such a structure on those organisms which are commonly held to be non-capsulated.

In the course of an investigation along another line this method has been tried on streptococci, staphylococci, members of the Gram negative coccus group, and many flagellate bacteria and has in no instance shown a capsule on these organisms although a similar structure may be demonstrated on all by means of a special technique which will be the subject of a subsequent report.

The advantages claimed for the above method of capsule staining are:

1. Standardization of the reagents employed.
2. The elimination of personal judgment as a factor by precise instructions as to the various steps.
3. Simplicity of technique.
4. Reliability of results.

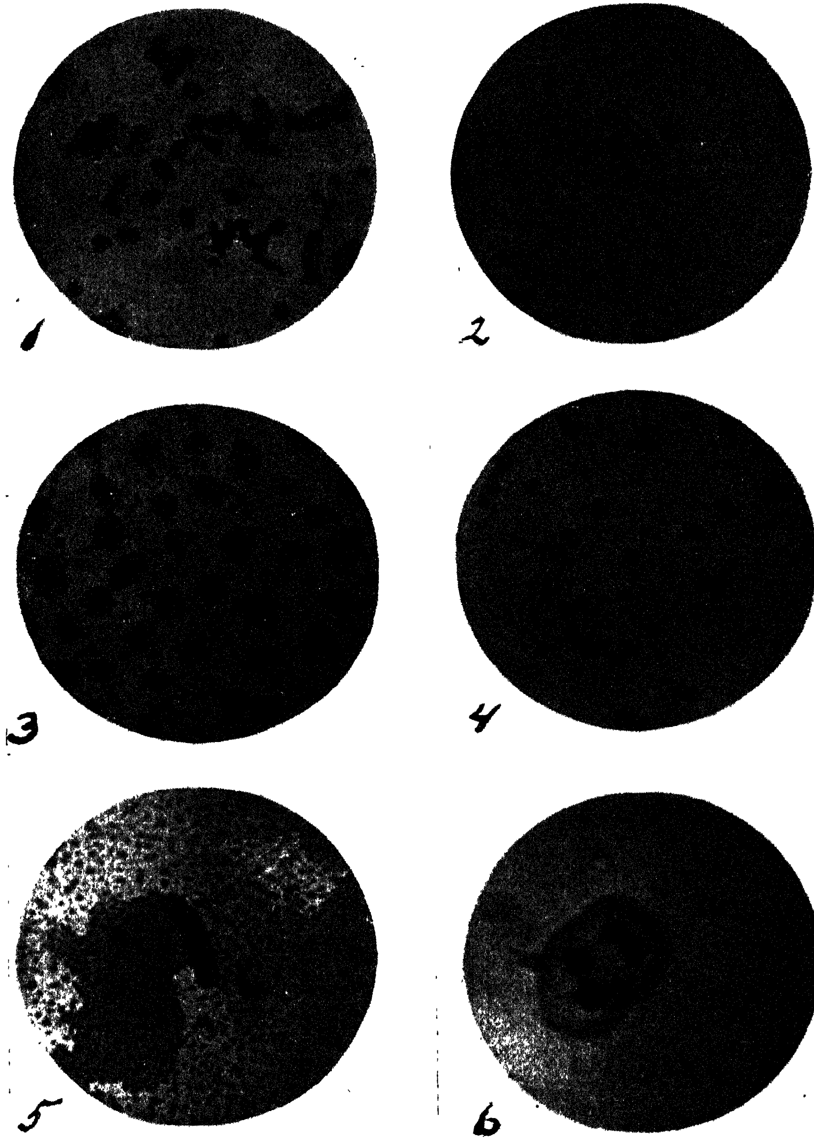

Fig. 1. Pneumococcus
Fig. 2. Streptococcus mucosus
Fig. 3. Pneumobacillus of Friedländer and Bacillus pyocyaneus
Fig. 4. Bacillus aerogenes
Fig. 5. Bacillus aerogenes
Fig. 6. Micrococcus tetragenus Showing the Indefinite Outlines of the Gelatinous Envelope

A CURIOUS ACCIDENT DUE TO B. PNEUMONIAE

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In the early summer of 1916 three barrels of soft soap were bought for use in scrubbing floors at the Ambulance de l'Ocean, La Panne, Belgium. These were placed in the storeroom of the Ambulance. About two weeks later one of the barrels exploded and its foaming contents ran out on the floor. During the next two or three days the other two barrels burst and the violently fermenting soap "boiled" out over the floor of the storeroom.

Bacteriological examinations were made of samples of the frothy soap from each barrel. Smears, made with some difficulty, and stained, showed great numbers of Gram negative bacilli approximately 0.8μ by 3 to 7μ. A few longer forms were also seen. Most of the bacilli occurred singly or in pairs, but chains of 6 to 20 elements were not infrequent. No spores were found.

Milk inoculated from the soap and heated to 80°C. for fifteen minutes remained sterile. Milk similarly inoculated, but not heated, showed in twenty-four hours coagulation with formation of a small amount of gas, but without any digestion of the curd.

On plain agar plates the colonies were practically all of one kind, round, 1 to 2 mm. in diameter, moist, elevated, semi-opaque, with regular edges. The growth strung away from the needle point when the colonies were fished.

Stab cultures were made from three similar colonies into tubes of glucose agar. The following morning the cotton plugs and part of the medium were found on the floor of the incubator, so violent had been the fermentation. Even plain agar slants inoculated at the same time were pushed up from the bottom of

the tube by the development of gas from the growth of bacilli in the water of condensation between the medium and the walls of the tube.

Three strains, designated "A," "B" and "C," were isolated. They showed some minor cultural differences, but were quite similar morphologically. The bacilli measured approximately 0.5 to 0.9μ in thickness by 2 to 6μ in length. In glucose broth strain "A" showed a tendency to grow in chains; but in most media all of the strains grew singly or in pairs. They were Gram negative. In milk cultures there was a more or less definite capsule. The organisms were non-motile.

On agar slants strain "A" produced a very vigorous, elevated, gray, translucent, spreading growth with regular edges. Smears from these cultures showed bacilli similar in all respects to those seen in stained preparations from the fermenting soap.

In plain broth there was uniform turbidity. The medium within forty-eight hours became very viscid and mucus-like, of that there was no tendency to sedimentation even after four weeks. There was a very slight annular scum.

Milk was rendered acid, a soft curd was formed and a few bubbles of gas were produced. There was no digestion of the coagulum.

The bacilli grew well in gelatin without liquefying it. A characteristic "nail-head" growth was not observed, although the growth on the surface was quite vigorous.

This strain fermented glucose, glycerine and mannite violently producing approximately 100 per cent of gas in a fermentation tube in twenty-four hours. In sucrose broth 80 to 90 per cent of gas was produced. Lactose was attacked less vigorously, but gas was formed to the extent of 50 to 60 per cent. Starch was not fermented.

In glucose broth this strain gave a Voges-Proskauer reaction. This reaction was not observed in lactose, sucrose, mannite or glycerine broths.

Strain "B" differed from the above in that it did not ferment lactose, did not coagulate milk, produced only 20 to 30 per cent of gas in sucrose broth and did not give the Voges-Proskauer

reaction. The viscidity of broth cultures was a little less pronounced. Strain "C" produced about 50 per cent gas in lactose broth but did not always coagulate milk, and did not give the Voges-Proskauer reaction. Otherwise it was similar to strain "A."

It is evident that of the three stains, "A" and "C" fall into Perkins'[1] Group I which ferments all sugars, while "B" belongs to Group II which ferments all sugars except lactose. Only one of the organisms (Cleveland 11) studied by Perkins fermented glycerine as vigorously as the three strains isolated from this explosive soap.

The chief interest in the organisms here described lies in the fact that they grew vigorously in a rather strongly alkaline cheap soap containing very little protein matter; that they attacked glycerine with remarkable avidity; and that the three strains, although isolated from the same source and belonging to the mucosus-capsulatus group of bacilli, showed slight cultural differences among themselves.

[1] Jour. Infect. Dis., 1904, **1**, 241.

THE FALLACY OF REFINED READINGS OF GAS PERCENTAGES IN THE FERMENTATION OF LACTOSE PEPTONE BILE AND LACTOSE BROTH

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The detection of the *B. coli* group in water, milk, oysters, and sewage effluents is of the utmost importance to the sanitary bacteriologist, for knowing its normal habitat to be the intestine of man and the higher animals, he at once infers that its presence must indicate pollution of intestinal origin. It is now firmly established that the *B. coli* group is an index of fecal pollution. Immediately after its discovery by Escherich in 1885, workers in this field of science began to devise methods of identifying the colon organism; as a result of their investigations we have various tests proposed and used quite extensively down to 1906, which aimed primarily at determining the presence or absence of the *Bacillus coli* group. The following tests were used:

A. Litmus lactose agar plate method (Wurtz, 1892)

This test is based on the principle that *B. coli* has the property of fermenting lactose with the production of acid. *B. coli* may be identified by the red colonies on the blue litmus lactose agar plates. The chief objections to this method are these: (1) Other microörganisms, notably the streptococci, also produce red colonies on litmus lactose agar plates. (2) Too much time is taken up in making supplementary tests for further identification.

B. Glucose broth-enrichment test (1901)

This test depends upon the fact that *B. coli* in glucose broth produces gas, which can be measured. In the early years of water bacteriology, this test was merely the first step in a long scheme of analysis; experience soon showed that 25 per cent or more of gas in the fermentation tube or in the small inverted vial was sufficient to justify the presumption of the presence of *B. coli*, and it now took the name "presumptive test." The chief advantage of this test is that only twenty-four to forty-eight hours are required to determine the presence of *B. coli*. The great disadvantage, however, is that it sometimes fails to show the presence of the colon group, since the culture medium is particularly suited to the development of other bacteria, which inhibit the colon group. Furthermore, it has been found that the factors of temperature, storage and reaction of the medium have much to do with this test.

C. Phenol broth (Reynolds, 1902)

The problem of the struggle for supremacy in the glucose broth tube was soon solved. By the addition of a small amount of a weak solution of phenol the ordinary water bacteria could be inhibited in growth to the advantage of the colon bacilli. Again, however, disadvantages arose even more serious than those affecting the pure glucose tube, since, in waters of fairly pure quality, phenol inhibits the growth of the *B. coli* group.

D. Eijkman test (1904)

Eijkman suggested that water forms might be eliminated in this test if the temperature of incubation was made 46°C. It was soon pointed out that weak strains of colon bacilli are also inhibited at that temperature.

E. Neutral red reaction (Rothberger, 1898)

This test depends on a color reaction. Objections have been made to its use because of the relatively large number of organisms unrelated to the colon group giving this reaction.

F. Lactose broth test

Experimental evidence is not lacking to show the usefulness of lactose broth as a medium for making the presumptive test for *B. coli*.

It has the decided advantage over the glucose broth medium that most water bacteria are inhibited to the advantage of the colon group; the fact to be emphasized here is that both these tests depend on the fermentation of sugars with the production of acid and gas.

G. Lactose bile test (Jackson, 1906)

It was not until 1906 that Jackson, working on this problem, suggested the use of a fermentation test such as had been used before in the case of the glucose and lactose broths but substituted, for these media, lactose bile. He believed that bile would most effectively represent conditions as the colon bacilli find them in the intestinal tract. Experiments soon proved the value of this test because: (1) The test can be performed in twenty-four to forty-eight hours. (2) Bile salts inhibit the ordinary water forms, and at the same time offer an ideal culture medium for typical *B. coli* (selective action). (3) Bile salts inhibit the growth of the glucose positive, lactose-negative fermenting organisms.

Of all these tests which have been offered, the lactose broth presumptive test and the lactose bile presumptive test have been used most extensively in the last decade. Against the test as a means of identification of the colon group the writers have no protest, but in its application in the laboratory, and in its interpretation, a change seems necessary.

It is a common practice in laboratories to read gas production in fermentation tubes and inverted vials very accurately, sometimes to the fractional part of a unit, thus: $33\frac{1}{3}$ per cent gas = + *B. coli*, $66\frac{2}{3}$ per cent gas = + *B. coli*, 75 per cent gas = + *B. coli*, etc. Of what value are these numbers, expecially fractions to the left of the equal sign to the practical laboratory man? He is primarily interested in what follows the equal sign. It is the purpose of this paper to show that the percentages of gas produced in lactose peptone bile and lactose broth have little quantitative value, and that there can be only one accurate way of reading and recording gas in these media, and that is, by positive (+) and negative (−) signs respectively. Among the factors influencing the percentage of gas production we have found: (1) Temperature, (2) time, (3) initial re-

action of the medium, (4) length of the inverted vial, (5) source of bile, (6) absorption of formed gas.

The changes and variations produced by these factors are so well-defined, as will become apparent to the reader, that refined gas readings are absolutely valueless in performing these presumptive tests for the colon group and results could be expressed more scientifically by the plus (+) and minus (−) signs. Our investigation was begun with the aim of studying only the lactose peptone bile test, but toward the end of the experiments it was deemed best to show that the same factors influence the readings in the lactose broth test, which is now being used in many laboratories. (Lactose peptone bile gives a precipitate of acid protein, which makes gas reading very difficult or impossible, and for this reason it is being dropped in making water analyses; lactose broth is taking its place.)

CULTURES USED IN THE INVESTIGATION

1. B. coli. Members of the *B. coli* group were isolated from Hudson River water, and cultures were kept in stock in the laboratory. The following characteristics are assigned to this organism: (1) short bacillus with rounded ends, (2) Gram negative, (3) non-liquefaction of gelatin in sixteen days, (4) grayish white growth on agar 20° C., (5) facultative anaerobe, (6) non spore-forming, (7) gas in lactose peptone bile, (8) fermentation of glucose and lactose with the production of acid and gas.

2. Hudson River water (taken from the river as it was needed in the experiments—from January 25 to June 10, 1916). Along with pure cultures of *B. coli*, Hudson River water was used, the purpose being to compare the action of pure and mixed cultures. Throughout the experiments, reference will be made to *pure* and *river* cultures. Various dilutions were used expressed as follows: 0 = 1 cc., 2 = 0.01 cc., 4 = 0.0001 cc. dilution.

METHODS

The culture media were prepared according to the Standard Methods of Water Analysis of the Laboratory Section of the

American Public Health Association (1912). The reaction, unless otherwise stated, was neutral to phenolphthalein. The greatest care was taken to have all the tubes uniform in size as well as in the amount of medium each contained. All were sterilized in the same manner, using 15 pounds steam for fifteen minutes.

METHODS OF DETERMINING THE AMOUNT OF GAS PRODUCED (PER CENT)

Vials of uniform size (2 inches long) were used in the greater part of the work. In accordance with the custom of reading gas percentages, a vial filled completely with gas, was read 100 per cent, and other readings made in proportion to the amount of gas in the inverted vial. In order to facilitate reading and at the same time to obtain a more accurate reading, a scale was constructed on a small card, which showed all the necessary subdivisions to enable the writers to read the percentages accurately; the card was held against the tube and the reading made.

RELATION OF TEMPERATURE TO THE AMOUNT OF GAS PRODUCED

Fifty 1 cc. portions of the 0, 2, and 4 dilutions of Hudson River water were inoculated into lactose peptone tubes. Twenty-five 1 cc. portions of a twenty-four hour nutrient broth of *B. coli* (0, 2, 4 dilutions) were also inoculated into another series of tubes. Four such sets of tubes were inoculated, making the total 900 tubes, and 225 were subjected to each temperature, there being four temperatures studied: 31°C. incubator temperature, 35°C. incubator temperature, 37°C. incubator temperature, 39°C. incubator temperature.

At the end of the twenty-fourth hour, the tubes were removed from the incubator and the percentages of gas recorded.

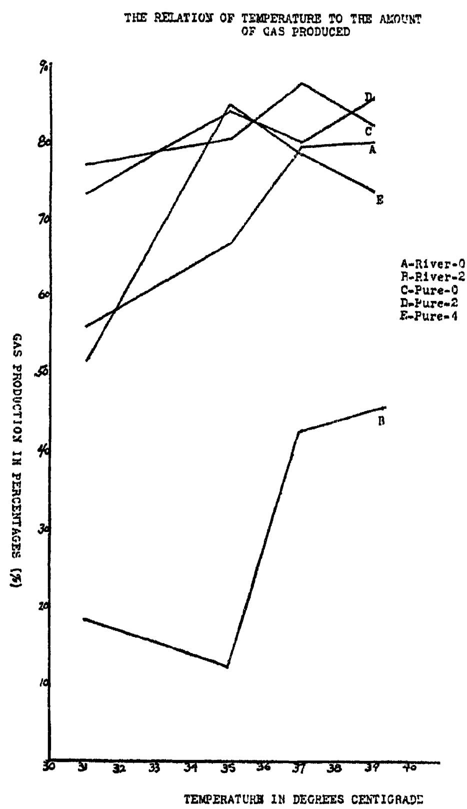
Reference to the plot of these results clearly brings out the variations caused by the temperature factor. The reader will

TABLE 1

Amount of gas produced at different temperatures by members of the B. coli group in pure and mixed cultures

TEMPERATURE	RIVER			PURE		
	0	2	4	0	2	4
°C.						
31	56.2% (20–95)	17.9% (0–55)	0%	77.8% (50–90)	73.0% (55–85)	51.6% (15–80)
35	67.5% (30–90)	12.0% (0–100)	0%	80.2% (65–90)	83.6% (70–90)	84.2% (60–90)
37	79.0% (35–100)	42.5% (0–90)	0%	87.5% (65–100)	79.3% (95–100)	77.9% (65–95)
39	79.9% (15–100)	45.6% (0–95)	0%	81.8% (65–100)	86.2% (75–100)	73.3% (60–90)

The results are the average of 50 readings in the case of the river cultures, and the average of 25 readings in the case of the pure cultures.

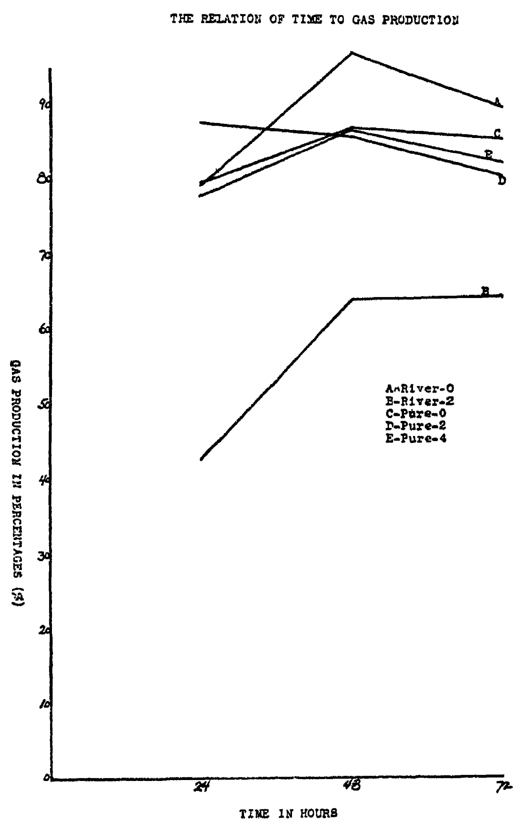


Plot 1

note the results under river 2, which are specially significant in that they show the widest range in gas readings when the same media and cultures were used.

RELATION OF TIME TO THE AMOUNT OF GAS PRODUCED

In this experiment, one cubic centimeter portions of the various dilutions of the pure and river cultures of the *B. coli* group were inoculated into lactose peptone bile tubes. Gas readings were made at the end of twenty-four, forty-eight and seventy-two hours. Incubation temperature, 37°C.



PLOT 2

TABLE 2

Amount of gas produced in different periods of time, by the B. coli group in pure and mixed cultures

HOURS	RIVER			PURE		
	0	2	4	0	2	4
24	79.0% (35–100)	42.5% (15–90)	0%	87.5% (65–100)	79.3% (45–100)	77.9% (65–95)
48	97.1% (50–100)	63.9% (0–100)	0%	86.0% (60–100)	87.0% (70–100)	86.9% (75–100)
72	89.9% (75–100)	64.3% (0–100)	0%	80.6% (65–100)	85.5% (65–100)	82.3% (70–100)

River culture results are the average of 50 tubes. Pure culture results are the average of 25 tubes.

It will be noted that the members of the *B. coli* group produce the maximum amount of gas at the end of the forty-eighth hour. The results show an absorption of gas beginning at the end of the forty-eighth hour. It will be seen that gas production is a variable, depending upon the factor of time.

THE RELATION OF THE LENGTH OF VIAL TO GAS PRODUCTION

The purpose of this experiment was to determine the difference in gas production (gas readings) when vials of different lengths were used. Vials from 1 inch to $5\frac{1}{2}$ inches in length were used, and the same amount of culture medium was added to each tube no matter what vial was placed in the tube. All the tubes were inoculated with 1 cc. of Hudson River water and incubated at 37°C. for twenty-four hours.

TABLE 3

Amount of gas produced in vials of different lengths

Vial length, in inches..	1	1.5	2	2.25	3	3.5	4	4.5	5	5.5
Readings in per cent...	95	70	61	62	51	43	58	61	70	41

The results in each case represent the average of five tubes.

The results should not be surprising; such have been the comparative results obtained continually in sanitary bacteriology. What we have done has been to choose arbitrarily a 2 inch vial to measure gas production—but is it not clear and evident that the length of the vial is an important factor in the end result?

RELATION OF THE INITIAL REACTION OF THE MEDIUM TO THE AMOUNT OF GAS PRODUCED

Tubes of lactose peptone bile and lactose broth, to which had been added varying amounts of acid and alkali were inoculated with 1 cc. of a twenty-hour nutrient broth culture of *B. coli* and 1 cc. of Hudson River water, and incubated at 37°C. Readings were made at the end of twenty-four and forty-eight hours.

TABLE 4

Amount of gas produced in lactose peptone bile, with varying initial reactions, by B. coli, in pure and mixed cultures

REACTION	RIVER		PURE	
	24 hours	48 hours	24 hours	48 hours
+1%	55.9% (35–90)	71.1% (60–80)	99.2% (60–100)	81.8% (70–95)
+2%	39.4% (5–80)	55.8% (30–75)	94.9% (30–100)	74.0% (65–90)
+3%	34.2% (5–70)	34.7% (5–60)	95.2% (50–100)	68.0% (55–80)

Results are the average of 35 readings.

TABLE 5

Amount of gas produced in lactose broth, with varying initial reactions, by B. coli, in pure and mixed cultures

REACTION	RIVER		PURE	
	24 hours	48 hours	24 hours	48 hours
+2.0%	0.0%	15.0% (10–20)	0.0%	0.0%
+1.5%	10.0% (5–20)	21.4% (10–30)	5.0% (5–10)	11.4% (10)
+1.0%	19.3% (15–35)	24.3% (20–45)	17.9% (10–30)	20.0% (15–30)
+0.5%	15.7% (10–20)	23.6% (20–35)	22.1% (20–30)	24.3% (20–35)
0.0%	20.7% (10–25)	29.9% (20–40)	29.2% (25–35)	30.8% (25–40)
−0.5%	21.4% (15–30)	29.3% (25–30)	27.5% (20–30)	33.3% (30–40)
−1.0%	23.3% (15–30)	32.5% (20–45)	35.8% (30–40)	46.6% (40–50)
−1.5%	16.4% (15–25)	23.6% (20–30)	32.7% (25–40)	45.6% (40–55)
−2.0%	11.4% (5–20)	39.2% (30–60)	22.9% (10–30)	55.8% (50–70)

These results are the average of 6 readings.

INDIVIDUAL VARIATION

The next point taken into consideration was individual variation. The best way to show that gas readings are fallacious

and have no real quantitative value is to present the variations not caused by any particular constant factor, but rather variations within a given group under similar conditions. Tubes of lactose peptone bile and lactose broth were inoculated with

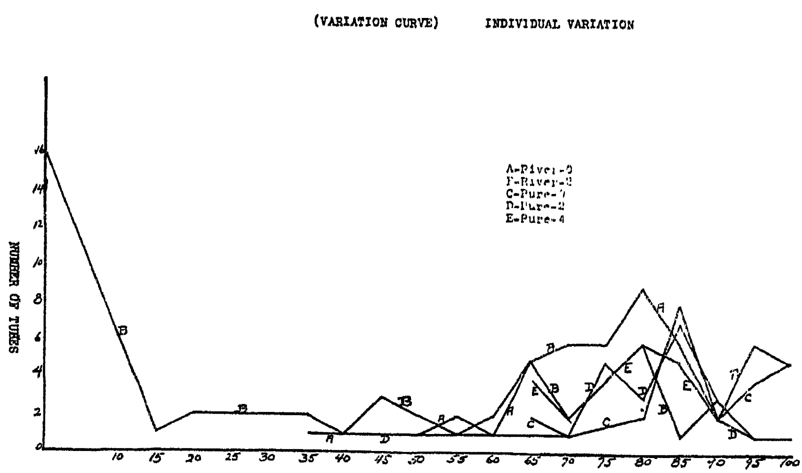
TABLE 6

Individual variation within the group (variation curve)

Bile medium

		PER CENT OF GAS																			
		0	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
River	0							1			1	2	1	5	6	6	9	6	2	6	5
	2	16		1	2			2	1	3		1	2	5	2	4	6	1	3		
Pure	0													2	1		2	8	2	4	5
	2									1	1			1	1	5	3	7	3	1	1
	4													4	2	4	6	5	2	1	
Lactose broth, medium																					
River	0		2	3	13	7	2														
	2		1	3	12	4	1	1	1												
Pure	0					10	10	4	1												
	2				1	3	14	3	2	2											
	4				3	6	4	3	4												

Temperature, 37° C.



PLOT 3

the pure and river cultures (various dilutions) and incubated at 37°C. for twenty-four hours, after which readings were made.

Reference to the table and to the plot showing the variation curve will indicate the wide range in the amount of gas produced. These results, perhaps give the most convincing evidence as to the utter uselessness of gas readings as quantitative values, since the same substance inoculated with the same organism gives such a wide variation.

RELATION OF THE SOURCE OF BILE TO GAS PRODUCTION

It was believed that bile from different cows might produce variations in gas production, and therefore, culture media were prepared using the bile from ten different cows. The culture tubes were inoculated with the pure and river cultures of the colon bacillus, and readings made at the end of twenty-four and forty-eight hours.

As soon as the bile was received from the slaughter-house, the following characteristics were noted: (1) acidity, (2) color, (3) consistency.

TABLE 7

BILE NUMBER	CONSISTENCY	COLOR	REACTION
			per cent
1	Clear, translucent	Light green	+0.5
2	Clear, translucent	Light green	+0.7
3	Heavy liquid, opaque	Green—black	+0.8
4	Slimy, slight yellow precipitate, opaque	Black	+0.5
5	Clear, translucent	Dark green	+——
6	Heavy liquid, green precipitate	Dark green	+0.8
7	Very heavy yellow precipitate	Dark green	+0.8
8	Slight precipitate	Dark green	+0.5
9	Slight cloudy precipitate	Dark green	+0.55
10	Clear liquid	Green—black	+0.55

TABLE 8

Amount of gas produced in the various biles

BILE NUMBER	RIVER	PURE	RIVER	PURE
	Twenty-four hours		Forty-eight hours	
1	39.0% (20–65)	63.5% (50–70)	Readings could not be made on account of a heavy yellow precipitate of acid protein.	72.0% (60–80)
2	70.6% (60–80)	81.0% (75–90)		85.0% (75–90)
3	53.0% (5–85)	72.0% (55–75)		82.0% (75–90)
4	40.0% (20–65)	69.0% (35–80)		81.0% (75–85)
5	29.0% (15–45)	65.0% (60–75)		72.0% (65–75)
6	40.3% (10–75)	84.0% (75–100)		94.0% (80–100)
7	42.5% (10–75)	82.5% (80–85)		86.0% (80–90)
8	25.6% (10–45)	48.0% (40–65)		73.0% (65–80)
9	29.4% (5–45)	68.0% (65–70)		79.0% (75–85)
10	21.3% (5–45)	42.5% (40–50)		63.0% (60–70)

CONCLUSIONS

From the data presented in this paper, it will be seen that:

1. The following factors cause variations in the gas readings in lactose peptone bile and lactose broth.

a. Temperature.
b. Time of incubation.
c. Initial reaction of the culture medium.
d. Length of inverted vial.
e. Source of bile.
f. Absorption of formed gas.

2. The gas readings in a given group vary within a wide range.

Since all these factors play an important part in the final gas readings, which necessarily must vary considerably, and since no real quantitative value can be assigned to any observation of gas production, under such conditions it seems that refined readings of gas percentages have no scientific justification in sanitary bacteriological analyses and that gas production could be recorded more conveniently and scientifically by positive (+) and negative (−) signs respectively.

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NOTE ON THE REACTION OF THE PARATYPHOIDS, ALPHA AND BETA, IN LITMUS MILK[1]

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Aside from the agglutination test, the most common method of differentiating between the paratyphoids, alpha and beta, is the reaction with litmus milk. Alpha is usually stated to give a permanent acidity while beta is said to give a permanent alkaline reaction after an initial acidity. Lehmann and Neumann (1912), for example, state that for *B. paratyphosus A*, litmus milk "bleibt sauer (*B. paratyphosus B* wird alkalish und blau.)" It is stated, moreover, that in litmus milk alpha is, "wie Typhus, ohne Aufhellung." While engaged in a broader study of the relationships of pathogenic milk-alkaline organisms as a whole, the attention of the writer was engaged by reactions of the paratyphoids which appeared to depart from the usually accepted type.

The data to which attention is directed are presented in the accompanying table, which shows the reaction of eight strains oi *B. paratyphosus A*, five strains of *B. paratyphosus B*, three strains of *B. enteritidis*, two strains of *B. suipestifer*, five strains of *Bact. pullorum*, and (for controls giving permanent neutral reaction to the time of evaporation) three strains of *B. avisepticus*.

Except for Cultures 5 and 8, the strains of *B. paratyphosus A* were received from Dr. Krumwiede of the New York City Department of Health. They were checked by agglutination tests and were found to correspond with other strains of *B. paratyphosus A*. Similar agglutination tests showed the approxi-

[1] Contribution 230 from the Agricultural Experiment Station of the Rhode Island State College.

TABLE 1

*Showing the reaction of certain strains of B. paratyphosus alpha and beta, and of some other bacteria, in litmus milk**

CULTURES	REACTION AS OBSERVED AT INTERVALS (DAYS) AFTER INOCULATION OF MILK TUBES																TRANSLUCENCY
	2	4	6	8	10	12	14	18	22	34	38	50	59	90	133	192	
B. paratyphosus α																	
5	+		+		+		+	+	N		N	N		– – –			–
8	N		N		+		+	+	N		–	–		– – –			–
24	+		+		+		+	+	N		N	N		–			–
27	L	+	+		+		+	+	N		–	– –		– – –			–
28	L	+	+		+		+	+	N		N	N		– – –			–
29	L	L	+		+		+	+	N		N	–		– – –			–
36	L	L	+		+		+	+	N		N	N		– – –			–
37	L	L	+		+		+	+	N		N	N		– – –			–
B. paratyphosus β																	
6	L		L	–					– –	– – –				– – – –			+
9	L		L	–					– –	– – –				– – – –			+
26	L		L	–					– –	– – –				– – – –			+
32	L		L	–					– –	– – –				– – – –			+
39	L		–	–					– –	– – –				– – – –			+
B. suipestifer																	
33	L	L	L	–	–		–	–	– –			– – –		– – – –			+
98	L	L	L	–	–		–	–	– –			– – –		– – – –			+
B. enteritidis																	
1	PR		CR				–	–	– –		– –			– – – –			+
25	PR		PR				–	–	– –		– –			– – – –			+
31	L		CR				–	–	– –		– – –			– – – –			+

TABLE 1—*Continued*

CULTURES	REACTION AS OBSERVED AT INTERVALS (DAYS) AFTER INOCULATION OF MILK TUBES																TRANSLUCENCY
	2	4	6	8	10	12	14	18	22	34	38	50	59	90	133	192	
Bact. pullorum																	
34	L	+	L	L			L	L	N	–	– –				– – –		+
56	L	+	L	L			L	N		N	–		– –	– – –	– – – –		+
59	L	+	L	L			L	N		N	–					– – – –	+
89	L	+	L	L			L		N		N	–		– –		– – – –	+
93	N	+	L	L			L		N			N		– –	– – –		+
B. avisepticus																	
48	N		N				N	N	N		N	N	N	N		N	–
52	N		N				N	N	N		N	N	N	N		N	–
83	N		N				N	N	N		N	N	N	N		N	–

* N = neutral; L = light; (+) = acid; (–) = alkaline. The degree of reaction is indicated by the number of signs.

mate unity of the paratyphoid B group, the hog cholera and the enteritidis groups. The strains of *Bact. pullorum* were derived from epidemics among young fowls and were checked by agglutination tests.

To present the matter briefly, the table shows that all of the "A" strains gave an initial acidity which returned to neutral by the twenty-second day; that the strains of paratyphoid B gave no initial acidity (though the tubes lightened somewhat), and gave an alkaline reaction beginning about the eighth day. By the time the paratyphoids A had returned to neutral, the paratyphoids B showed a moderately strong alkaline reaction (grade 2). The hog cholera and the mouse typhus strain behaved as did the paratyphoids B. The enteritidis strains agreed fairly well with the paratyphoids B, but gave a partial or complete reduction on or before the sixth day. The tests of enteritidis will bear repeating. The pullorum strains showed an initial acidity which passed quickly into a neutral, then to an alkaline

reaction about the thirty-eighth day, sometimes earlier. The strains of *B. avisepticus* did not alter the appearance of the milk in any way during the course of the observations and the control tubes remained essentially unchanged up to the time of evaporation.

The special point to which attention is now called is that notwithstanding the difference which appears between the paratyphoids A and B at the twenty-second day, the difference tends to disappear with the lapse of time. On the thirty-eighth day two of the eight paratyphoid A strains were alkaline; on the fiftieth day three were alkaline; and on the ninetieth day all showed a strong alkaline reaction (grade 3). At this time the only difference between the alpha and beta types was that the beta types showed a slightly stronger alkalinity (grade 4), also by *transmitted* light, a deep wine-red translucency., while the A types showed no translucency. In other words after the lapse of ninety days in litmus milk, the two groups were practically indistinguishable so far as the degree of alkalinity was concerned.

These results appear to suggest that "permanent acidity" cannot be regarded as one of the chief characteristics of *B. paratyphosus A* as stated by Bainbridge (1909); and that initial acidity cannot be regarded as a permanent feature of *B. paratyphosus B* as stated by many writers. It is clear that *both* give the "terminal alkaline" reaction; and the only difference is one of time and degree. In other words there appears to be, in this respect, no fundamental *qualitative* difference between the two bacterial types, but one involving *quantitative* relations only. This circumstance might suggest that, although the litmus milk test may be employed with advantage to differentiate between the two groups, the readings should be taken at a definite time, possibly not earlier than the twelfth day; and for *B. paratyphosus A*, not later than the thirtieth. In other words the test of *B. paratyphosus A* in litmus milk is not the absence of a terminal alkaline reaction, but the presence of a marked initial acidity; while the test for *B. paratyphosus B* is a strong alkaline reaction which usually begins to appear in

milk at a period comparable with the acid-stage of *B. paratyphosus A*. In view of the similarity in reaction after long periods, the translucency ("Aufhellung") test would seem to gain an added significance as a means of diagnosing the A types, although more data are needed on this point.

ADDENDA

After the above note was prepared for press, the paper by Krumwiede, Pratt and Kohn (1917) came to the notice of the writer. The results for the most part correspond, aside from the circumstance that the present writer did not observe the intergradation of the A and B types, with respect to the degree of the alkaline reaction. This may be due to the fact that Krumwiede studied a much greater number of cultures. Krumwiede does not mention the presence or absence of translucency which the present writer is inclined to regard as an important diagnostic feature, separating the A types from the B and enteritidis and hog cholera types quite as distinctly as the initial acid reaction of the A group. It might be of interest to ascertain whether the feature of translucency, if studied in a greater number of cultures such as Krumwiede reports, would show intergradations which the present writer may have missed in the smaller number of strains examined.

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A STUDY OF THE DIPHTHEROID GROUP OF ORGANISMS WITH SPECIAL REFERENCE TO ITS RELATION TO THE STREPTOCOCCI[1]

PART II. CLASSIFICATION OF THE DIPHTHEROID GROUP

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PREVIOUS CLASSIFICATIONS

The difficulties of biologic classification among organisms showing such slight differentiation as the bacteria, have long been noted by bacteriologists. Attempts to arrange assemblages of such organic forms under the captions of species have not met with uniform success, partly on account of overlapping characteristics, and partly on account of the various transitions which this subjective term "species" has experienced from time to time. It is much more practicable to take refuge behind the word "group" as Dr. Prudden (1898) long ago remarked.

Biologically it is doubtful if toxin production on the part of an organism should be considered as a major characteristic, nevertheless from the standpoint of pathology and medicine, it is a quality of first importance, and thus considered indicates a position "sui generis" for the Klebs-Loeffler bacillus. I merely cite this as a concrete difficulty. An adequate classification must conform to the biologic conception, yet it should defer as far as possible to the more utilitarian claims of practical medicine and pathology.

There have apparently been very few attempts to develop a comprehensive, systematic arrangement of the diverse members of the diphtheroid group. The term pseudo-diphtheria

[1] A thesis for the degree of Doctor of Public Health.

bacillus has been inadvertently applied to all atypical diphtheria organisms. This is generally recognized as unfortunate, as the term was already preempted for the organism described by Loeffler (1887) and Hoffman-Wellenhoff (1888) and now commonly known as *B. Hoffmanii.*

For some years after the discovery of this bacillus there followed a controversy regarding its relation to the Klebs–Loeffler organism. The monists held that the Hoffman bacillus was an attenuated variety of the true diphtheria bacillus, while the dualists regarded them as distinct and separate species. It cannot be said that the question is fairly settled yet, although the present trend of opinion is decidedly opposed to the view that the organisms are separate species in the sense of being totally unrelated. In view of the immense amount of work that has been done, the contention of Flexner and others that *B. Hoffmanii* or the pseudo-diphtheria bacillus is a true mutant seems logical.

It was soon apparent that the pseudo-diphtheria bacillus was by no means a single race of organisms, and that in all probability there existed a large number of strains of diphtheria-like bacilli, which are identical with neither the Klebs-Loeffler nor the *B. Hoffmanii.* Although there have been desultory contributions to the literature of this subject for the past twenty years, bacteriologists in general have lapsed into a state of apathy regarding the pathogenic properties of diphtheria-like microörganisms. True, this negligent attitude has been unsuccessfully assailed from time to time, by workers whose experience with the group had convinced them that all its members did not merit a wholesale relegation to the saprophytic scrap-heap. The investigations of Alice Hamilton, Ruediger, Hektoen, Rosenow and others have demonstrated beyond peradventure, that certain strains of this diverse group are capable under certain conditions of showing definitely pathogenic properties.

The lethargic attitude regarding the diphtheroids suffered a shock as the result of a series of investigations begun by Frankel and Much in 1910 and continued by de Negri and Miermet, Bunting and Yates, Rosenow and many others. The first

mentioned authors studied exhaustively thirteen cases of Hodgkin's disease, and were able to recover from the sediment of the lymph glands previously treated with antiformin, certain pleomorphic non-acid-fast bacilli which they did not succeed in cultivating. Frankel and Much, as a result of their studies came to the conclusion that this organism was either an attenuated or a special variety of the tubercle bacillus.

Shortly after this paper appeared, de Negri and Miermet and later Bunting and Yates succeeded in cultivating this organism which had the characteristics of a diphtheroid, and was named by the latter authors *B. corynebacterium Hodgkini.* They claimed to have reproduced with it the characteristic lesions of Hodgkin's disease in monkeys although as yet the contention lacks confirmation. As a result of these investigations a new interest has been awakened in this group, which has manifested itself in the character and number of contributions to the recent literature.

These contributions have been directed mainly toward one of the more pressing exigencies of the situation, namely, the discovery of the *B. Hodgkini* in normal and non-Hodgkin's lymph glands as well as in other locations. As a result a large number of diphtheroids of various types have been found, but owing to the lack of any classification of the group considered as adequate, the various observers state with one accord that they are unable to harmonize their various findings with those of each other. Arbitrary groupings and provisional classifications, based largely on expediency or on some minor characteristic have failed to bring order out of the confusion which at present reigns.

B. xerosis also labors under a variety of interpretations. Some make this species identical with the avirulent diphtheria bacillus, while the term is restricted by others to the Kuschbert-Neisser (1884) organism originally thought to be the cause of xerosis, but later found to be a more or less common inhabitant of the normal conjunctival sac.

All through the literature one finds instances of diphtheria-like bacilli receiving specific designations based on a variety

of minor characteristics, such as morphology, or action on gelatin, or perhaps on potato, or pigment production, appearance on serum, or some inadequate combination of these characters, many of which can easily be modified. Another source of confusion has been the results of the sugar reactions in the hands of various observers. For a long time strains were tested on glucose only and naturally it is difficult to correlate such descriptions with those which have been based on six or eight different sugars. Little attention has been paid to immunologic characteristics as a criterion for classification.

The best classification of the diphtheroids that I have encountered is undoubtedly the one made by Morse (1912 a). She divides the entire diphtheria family into two main groups: the Klebs-Loeffler bacillus and the diphtheria-like bacilli or diphtheroids. Of these latter she recognizes four sub-groups which are the following:

Group A. This is the largest numerically. The bacilli correspond to the "organism x" described by Hoag from the Danvers State Hospital. It is a medium sized bacillus, showing solid, barred and wedge forms, with abundant but small and imperfect granules. On serum it produces a heavy, confluent, glistening growth with a characteristic salmon-pink color. It ferments glucose and sucrose, but not maltose or glycerin.

Group B. The organisms of this group are usually larger than those of Group A, and thick forms with clear cut bars predominate. Neisser's granules are very large and irregular. The growth on serum is heavy and varies in color from white to yellow. It is often noticeably dry and granular. Glucose is always fermented, maltose and glycerin usually, but not sucrose.

Group C. This, the smallest group, is differentiated primarily by its slow, scanty, colorless or white growth. Morphologically, the organisms resemble those of Group B. They always acidify glucose, and both sucrose and maltose usually.

Group D. Composed of thick, small, straight bacilli, often barred and wedge-shaped, showing no granules. The growth on serum may be scanty or abundant and is white or yellowish white in color. They do not act upon glucose, maltose, glycerin, or sucrose.

Morse names these four groups as follows: "Group A, *B. Hoagii:* Group B, *B. flavidus:* Group C, *B. xerosis* and Group D, *B. Hoffmanii.*" I have retained this terminology in my own classification, and have confirmed the validity of these various groups as they stand.

DISTRIBUTION OF THE DIPHTHEROID GROUP

The diphtheroids are among the most ubiquitous of all bacterial organisms. They are to be found not only in the bodies of animals and man, but also have a wide distribution in nature. R. O. Neuman (1902) and Sudeck (1896) first found them in the air. Wade and Harris (1915) and Torrey (1916) have made definite experiments on this point with positive results. Wade exposed 18 blood-agar plates to the air and recovered 45 colonies of diphtheroids. He has also isolated these organisms from the urine and feces. I have myself isolated them from the air of various laboratories. McNaught (Graham-Smith, 1908) has obtained them from water. McCambell (Graham-Smith, 1908) and Bergey (1904) have found them in milk.

It is of great interest to know that diphtheroids have been found in practically all the organs of the human body in either pure or mixed culture, and in both health and disease. Since the claim of their etiological relation to Hodgkin's disease, there have been several reports regarding their presence both in normal lymph glands and in gland conditions bearing no relation to Hodgkin's disease. Harris and Wade (1915) have written a report on the "Wide distribution of diphtheroids and their occurrence in various lesions of the human tissues." Besides normal and pathological lymph glands (not Hodgkin's) they have isolated diphtheroids from leiomyomata, fibroma and hepatic cancer, as well as from tuberculosis of lymph glands and lymphosarcoma. Fox (1915 b) has isolated them from the normal and diseased lymph nodes in many cases. Several strains came from lymph glands draining enlarged joints. Torrey (1916) in a special article on lymph gland bacteria, cultured 30 strictly lymph gland conditions and 10 conditions in which

the lymph glands were secondarily involved. He recovered the organisms from one type or other in 22 of the cases. Bloomfield (1915) reports a similar experience. Hoag (1907) finds the organisms in almost every part of the body but mainly in the respiratory tract. Bergey (1904) has found them in spontaneous abscesses in animals, and in cutaneous suppurations and in tumors. Hoag (1907), and Orr, Rows, and Robertson (1910) found these organisms in the central nervous system. J. A. Langford (1914) and Bunting and Yates (1914) found diphtheroids in the spleen in Banti's disease. Hektoen (1901) found them in large numbers in the liver, Axenfeld (1899) and others in the conjunctival sac, Walsh (1899) in the skin, Hall and Stone (1916) in the lymph glands of horses, sheep and calves. They have been found by many observers in the urethra (Hine, 1913) in the vagina (by Voigt, personal communication) and in the bladder (Townsend 1905). Rosenow (1915) has isolated them from the blood, joints, glands, lungs, skin, parotid gland, ganglia of the central nervous system and many other locations. I have myself isolated them from many locations in the body, but especially from the upper respiratory tract, skin, urethra, and lymph glands, less often from joints, blood, lungs and spinal fluid. This list could be indefinitely extended, but as no purpose could be served by so doing, I shall briefly summarize this topic by saying that the most common situations in which diphtheroids may be found in the human body are the mucous surfaces and skin, but a perusal of the references cited shows that no portion of the organism is exempt from them.

MORPHOLOGY

The size and shape of diphtheria-like organisms is subject to an almost infinite number of variations. Some of the subgroups have a fairly constant shape while in others the pleomorphism is so marked that almost any conceivable shape may be assumed as the environmental factors are altered. The detailed classification of diphtheria bacilli by Wesbrook includes all the ordinary and many of the rarer forms encountered in

the throat. The shapes most frequently met with are embraced under his three divisions; first the granular, second the barred and third the solid type. Type I shows spherical or oval metachromatic granules, usually located at the ends of the bacilli although they may be noted elsewhere. The protoplasm stains faintly (Loeffler's). Type II is the barred or segmented type and is characterized by great regularity of staining. The bars may be so arranged as closely to resemble streptococci and indeed may be mistaken for them. Cobbett (1901) designates this form as a separate morphological type. Neisser (1897) mentions a streptobacillus which is not unlike the Klebs-Loeffler organism. This streptococcoid form is not one that has received much recognition in the literature, but in the light of the relation between the streptococcus and the diphtheroid groups to be discussed later on, I believe that such forms merit a more careful consideration. The number of the segments or bars may be from 3 to 20 and they may or may not be metachromatic. The third or solid type is non-granular, may have almost any size and shape, and often appears as a diplobacillus. The different types ordinarily vary in thickness from 0.25 to 2 microns and in length from 1 to 8 microns.

The varied morphology of the diphtheroid group and the conditions which modify it, have been the subject of much discussion in the past. The question has hinged largely on the stability of the morphological and toxic types under varying conditions. A. Williams (1902) makes a thorough revision of this whole subject and in addition repeats the previous experiments as well as contributes new ones with a view to clearing up the question as to the fixity of biological types. Her conclusions are as follows:

Though some cultures change on some media, each changes in its own way and each culture still has its own individuality. Even though the morphology of a culture may be radically changed by alteration of media, etc., nevertheless when transplanted to the same media and under the same conditions that conduced to the establishment of the type, the original morphology will return.

Morse (1912) also believes that the morphological individuality of a culture is retained tenaciously under good conditions. She studied 295 strains and was able to correlate definitely several morphological types with other fixed characters such as sugar fermentation, etc. Both authors say however that the strain must be observed through several generations and a constant morphology obtained, before attempting cultural changes to induce marked pleomorphism.

My own observations are for the most part in accord with these workers although there is one sub-group which has a pleomorphism so extreme that it will not conform to the above restrictions. Among the strains that I have studied there is a certain percentage described as *B. enzymicus* in Part I (Journal of Bacteriology, March 1917) which undergo a very remarkable change of form which remains quite constant and which is not brought back to type merely by restoring the original conditions. A barred, fairly long bacillus of this kind can be changed to a typical diplococcus or to a long-chained streptococcus (diplo-streptococcus, Part I, pp. 84–85, Journal of Bacteriology, March, 1917). When this form has developed it remains quite constant and although it is possible to cause it to assume, a bacillary form again, this is accomplished only with great difficulty. These forms are not involution forms in the ordinary sense of that term and are to be clearly distinguished from the ordinary coccoid form of diphtheroids and other bacilli. The coccoid forms are usually single, vary wonderfully in both size and staining characters and are almost always metachromatic. They take the stain very intensely and irregularly, and generally appear under rather adverse conditions. One usually has no difficulty in distinguishing them from true cocci. The streptococcus form is not distinguished from the true cocci by ordinary staining methods. Some of the strains which I have studied showing these characteristics are numbers 1, 3, 11, 13, 14, 16, 26, and 33 (see table 2).

INVOLUTION

This is a very common phenomenon among the different members of the diphtheroid group, being most frequent and

most varied among the granular and barred types, while it is more unusual among the solid types, although some of them show it. Bunting (1913), de Negri and Miermet and many others emphasize the extreme pleomorphism of the so-called *B. Hodgkini.* Club forms and segmented forms are common, while almost all observers agree that the coccoid forms are so confusing that it is only by repeated platings that one may be sure that a pure culture is obtained. Fox (1915) shows photomicrographs of diphtheroids from the lymphatic glands, mostly of Hodgkin's cases. Some of these develop long clubbed metachromatic forms, others assume very long sinuous filamentous forms. Others occur as threads, some resemble short chains (streptococcoid forms) while some are fused and branched.

Neisser (1884) and Eyre (1896) speak of the marked clubbing of the xerosis bacillus on dried glycerin serum. Rosenow (1915) mentions the remarkable involution forms of the causative diphtheroid of erythema nodosum. In ascitic-glucose broth only bacillary forms appeared, while on blood-agar plates, he recovered small diplococci in short chains.

Strain 1 in the series under investigation in this study showed very similar characteristics. In ascitic glucose broth, growth was slight with no clouding of the media. No coccoid forms developed under these conditions. The bacilli were barred and granular. The granules stained intensely with Loeffler's stain while the protoplasm stained faintly. Branched forms were numerous after four days, and on several occasions the most extraordinary filamentous network was seen; this branching form of the organism resembled closely certain of the streptothrices. On the nodal points of the network one or more granules of heavily staining chromatin were to be seen. These granules were of varying size and often stained metachromatically. The network itself stained faintly, and resembled debris but on closer examination and differentiation proved to be a direct outgrowth of the organism. The shorter fused forms described by Park and Williams (1910) were also not uncommon in this medium and were usually associated with the more complex structure just described.

On blood-agar slants the ordinary coccocid forms developed. The size of the cells varied between wide limits. They grew singly or in clumps, seldom as diplococci and were spherical or ovoid in shape. They stained intensely in Gram or Loeffler's stains and metachromatism of varying grades was the rule. With careful differentiation or double staining, marked chromatic irregularities appear. A more complete description of this strain will be found in Part I, Journal of Bacteriology, March, 1917.

GROUPING

Most characteristic among the various possibilities of arrangement is the palisade grouping. This was described by the early writers Neisser (1897), Prochaska (1897), and others, and has been emphasized by the more recent contributions to this subject, Park and Williams (1910), Teoumin (1913). V-shapes and diplobacilli are also very common. It is also usual to see diphtheroids in clumps or in tangled masses.

STAINING

As a general thing diphtheria like-organisms stain readily and intensely with the simple anilin dyes. Occasionally one encounters a strain of granular bacilli which reacts faintly to Loeffler's but in such instances Gram's stain produces excellent results.

With Gram's stain one finds all gradations of reaction. It is usually stated that all diphtheroids are Gram-positive. Prochaska (1897) says that "decolorization must not be too energetic." Hamilton (1907) describes a Gram-negative diphtheroid. De Witt (1912) speaks of Gram-variable diphtheroid bacilli. I have referred to the effect of old or dry media, the age of the culture, etc., on the intensity of the Gram stain with the so-called *B. Hodgkini*, (Mellon, 1915). "In some cultures all grades of reaction to this stain might be seen; in others, the entire culture was finally decolorized." "Coccoid forms usually take the stain more intensely than the bacilli." This has also been observed by Steele (1914) and others.

No matter how pleomorphic a strain may be, the chromatin granules are practically always Gram-positive, while the body of the bacillus or projections therefrom are usually Gram-negative, if the culture is relatively young. The filamentous forms which I have described above as well as in another communication, are always Gram-negative (Mellon, 1915). These variations are much more noticeable in the granular and barred types than in the solid ones. So although the diphtheroids can properly be classed among the Gram-positive bacilli, the limiting conditions must be very strictly adhered to, and even then some strains will be doubtful.

The principle holds equally well regarding the property of "fastness" to acids. Diphtheroids are usually described as being non-acid-fast, and as a general proposition this is true. Nevertheless Wade and Harris (1915) believe that some strains are acid-fast. They class *B. smegma* among the diphtheroids and its reaction in this regard is well known. In addition they have isolated a diphtheria-like organism from the mesoappendix which liquefied blood serum and gelatin, was Gram-positive and acid-fast. Wolbach and Honeij (1914) report "partial acid fastness of a diphtheroid isolated from leprosy lesions." The granular portions of these bacilli are fast to 2 per cent H_2SO_4 followed by 95 per cent alcohol; and 3 per cent HCl in 95 per cent alcohol, reagents acting for 30 seconds. Similar treatment completely decolorized the Klebs-Loeffler and mixed cultures of cocci and bacilli from the throat.

Kedrowski (1901), Bayon and Williams (Harris and Wade, 1915) have cultivated diphtheroids from leprosy, each regarding his culture as a stage in the life cycle of the Hansen bacillus. Kedrowski assumes a mutation from a non-acid-fast to an acid fast organism because he recovered the latter after injection of the former. Campana and Babes (1910) thought Kedrowski was working with mixed cultures since they could not confirm his results. Duval and Duval and Harris also quoted by Harris and Wade have not been able to repeat these experiments. Dr. Eggers tells me that Kedrowski's work has been recently confirmed. Out of 45 colonies of diphtheroids on blood-agar plates

exposed to the air, Harris and Wade (1915) recovered 7 which were distinctly acid-fast.

I have experimented with quite a number of strains in this regard. I used as a routine 1 per cent HCl in 20 per cent ethyl alcohol and 2 per cent HCl in 50 per cent ethyl alcohol as a decolorizing agent. My results are tabulated in table 2. Out of 37 strains treated with 1 per cent HCl in 20 per cent alcohol for one and one-half minutes following previous treatment with carbol-fuchsin, I found that 13 strains retained the stain very definitely. The remaining strains were either decolorized or doubtful, and seven were completely decolorized. Six strains resisted 2 per cent HCl in 50 per cent alcohol. The granules held the stain tenaciously but often the body of the organism was pink also. The results were fairly constant on Loeffler's medium and 2 per cent glucose-agar but all strains that would grow on potato were either negative or doubtful. More constant results are claimed by Rehr (1915) using alcohol-acetone to decolorize the bacilli previously stained by Gram. He claims to be able to diagnose a much larger percentage of cases of true diphtheria by this means.

Here again is seen a lack of constancy in reaction, depending not only on the individuality of the strain but also on the chemical constitution of the medium. My experience with the staining properties of this group gives me the impression that the diphtheroid protoplasm is very labile, very dependent on factors of which we know little, and which we cannot control. For this reason I am rather skeptical about drawing very definite conclusions of any kind from evidence which has as its basis the retention of a stain by such an ephemeral matrix.

The polychromasia or metachromasia of various members of the diphtheria group has long been known and commented upon. It is quite characteristic for members of the granular group to exhibit reddish or magenta colored chromatin. Under certain conditions these granules may become a very brilliant red even when a pure Loeffler's stain is used. A. Williams (Park and Williams 1910) believes "that the metachromatic masses occurring in involution forms of *B. diphtheriae* represent

a primitive sexual process, a sort of autogamy."[2] She believes that disturbance of the culture at intervals facilitates this process. I have been able to verify this observation although it occurred as a result of routine examination of a culture in broth. Strain number 16 of my series grew faintly in ascitic broth and only in the bottom of the tube A transplant was made October 23, and examined October 24, 26, and 29, 1915. No metachromatism was noticed. On October 29, I shook the tube vigorously to try to induce a heavy growth, which just began October 30. At examination at this time showed the large clubbed and branched forms and the immense bright red granules above referred to. When examined on an agar hanging block these forms showed very active growth and a fusion of the metachromatic granules which led Williams to interpret the process as a primitive sexual one.

FIG. 1

Strain 29 of my series also showed a remarkable series of pictures covering but two microscopic fields. The transplant was made to fresh potato, which developed a very granular organism from one that had the chromatin concentrated in the ends of the bacillus and which did not resemble the Klebs-Loeffler bacillus. It was stained in carbol-fuchsin and decolorized in the weak acid-alcohol above referred to and very lightly counter stained with weak Loeffler's. The very remarkable forms shown above were found at about one place in the cover-glass, due I believe to the fact that I barely touched the end of the wire on a very moist growth and drew it across the cover-glass but once. These forms are surely very suggestive of the karyokinetic figures so common in the higher forms.

MOTILITY

None of the cultures in my series showed motility and I have been able to find only one instance recorded in the literature.

[2] Schaudinn has shown a primitive conjugation in *B. Bütschlii*.

De Witt (1912) describes one such organism which she proved to be the cause of the pathologic condition from which it was isolated.

CAPSULES. SPORES

I have demonstrated capsules in my strain number I, isolated in coccus form from an animal. I have not been able to find any reference to such in the literature. There are many comments on the absence of capsules, spores and motility among the diphtheroids.

CULTURAL CHARACTERISTICS

Glucose-agar

The appearance of the colonies on this medium varies with the subgroup and very often with different strains of a subgroup. *B. xerosis* exhibits a dry, transparent film which gives the slant a ground glass appearance. This is not changed on prolonged incubation or by standing at room temperature. All observers agree on this point. *B. flavidus* also has a dry granular growth which may or may not be pigmented, but is usually much more luxuriant than *B. xerosis*. These are the only two subgroups of this species to give a dry growth.

Typically, diphtheroid colonies are very moist. Prochaska (1897) describes precise, whitish-grey colonies, which enlarge rapidly and form an elevated growth. They are dark in the center and clear on the border, which may be serrated. When pigment is present it varies from light or lemon-yellow, to orange or red. Some have a brown pigment. Hamilton (1904) has described a strain giving a purple color on agar. Prochaska (1897) calls attention to the fact that as the medium dries slightly the center of the colony may fade. My strains 4 and 5 faded out completely, even though the tubes were sealed. The growth became perfectly transparent. This is not a usual feature. Some strains refuse to grow on serum-free media when first isolated but can usually be trained to do so. The more saprophytic subgroups, *B. Hoagii* or *B. diphtheroides-liquefaciens*

give an abundant moist, coalescing growth which proceeds slowly at room temperature.

Blood agar

This medium is conducive to more luxuriant growth of all strains. The typical colony has a dark center and light border. Pigment is more likely to develop on blood-agar. Not infrequently the colonies are stippled. *B. flavidus* gives a yellowish, spreading, adherent growth which is wrinkled or corrugated radially. Some strains show concentric wrinkling. An occasional race is hemolytic. The bacillius of Preiz-Nocard and of Hall and Stone (1916) was markedly so. My strains 13 and 14 were slightly hemolytic. It is not uncommon for a blood-agar plate to become brown from the acid produced. Not infrequently the colony takes up the changed hemoglobin leaving the surrounding medium colorless.

Blood-agar plates offer a ready means of determining the purity of a culture on account of the various forms of colonies that develop. I have been able to separate different types of organisms by the type of colony produced. This is by no means an infallible criterion, since the variants of a pure strain may show different types of colony. It is often necessary to make several rapid transplants, when the colonies will then be alike in case the strain is pure.

Loeffler's blood serum

The various members of the group grow well on this medium, giving as a rule, moist, transparent or opaque colonies with the fastidious strains, and heavy, opaque, confluent growths with the saprophytic ones. There is little opportunity for colony differentiation with this medium. It is usually the best medium to indicate proteolytic change. De Witt (1912), Hamilton (1904), Wade and Harris (1915) and Graham-Smith (1908) have all described proteolytic strains of this group. One strain of my series (32) was a rapid liquefier of serum.

Gelatin stab

The majority of races will grow in gelatin, some of them quite luxuriantly, although it is the exception to have liquefaction occur. Most of the liquefiers are found in the subgroup *B. diphtheroides-liquefaciens*. Strains 4 and 5 of my series caused a slow liquefaction although they belonged to the *B. Hoagii* subgroup. Pigment formation is not uncommon in gelatin. Viability is well preserved. Strain 3 was alive after eighteen months on this medium. Ordinarily one sees a greyish-white growth which not uncommonly extends along the entire line of inoculation, inasmuch as this group readily adapts itself to anaerobic conditions. Strain 14 developed single, thin, spreading colonies along the upper third of the stab. They were dark in the center and had a lighter serrated border. Strain 21 developed a "nail-head" culture for a few days, then a funnel shaped liquefaction along the entire line of inoculation. Strains 31 and 42 grew mostly in the bottom of the tube showing their preference for anaerobic conditions.

Broth

Here again one finds great variation although there are two main types of growth; first, the formation of scanty, granular, flocculent or nebulous material that collects along the sides or bottom of the tube after twenty-four hours leaving a clear supernatant; second, the heavy, luxuriant, diffusely turbid growth, depositing a finely granular or mucoid sediment. Certain races develop a thin, friable pellicle which is deposited as a flocculent sediment on slight agitation of the tube. *B. xerosis* and *B. flavidus* more often give rise to a pellicle as they are more closely related to *B. diphtheriae* than the others. *B. enzymicus* grows very scantily in broth although it can be made to grow luxuriantly by the method described in Part I, Journal of Bacteriology, March, 1917. The broth has a foul or sour odor depending on whether acid or alkali is produced. De Witt (1912) alone has ascribed a peculiar odor to her cultures. She says that it resembles acetone, and was identical with that coming from the

lesions of the patient. I have observed a very unusual odor with strains 2, 11, 13, and 14. It is always noticed when a carbohydrate is fermented, and it resembles the odor of the esters of the higher alcohols. It resembles amyl alcohol very closely or the heavy sweet odor that comes from lilies. The fact that strains 13 and 14 were isolated from a case of gonorhoea suggests the idea that these organisms may be a factor in the repugnant odor of a case of this kind. It is known that the odor of the soil is often due to bacterial activity. It is interesting that all these strains belonged to the *B. enzymicus* subgroup.

Litmus milk

A majority of my strains caused but little change in this medium. The Ruediger bacillus (Ruediger, 1903) completely decolorizes litmus although no acid is produced. This feature constitutes the most noteworthy cultural characteristic of the organism. This is probably a reduction change. Several of Fox's (1915) gland diphtheroids reduce litmus. Strains 21 and 1 in my series exhibit this action. Graham-Smith mentions this change in his *B. diphtheroides-liquefaciens*. It most commonly occurs in saprophytic races producing alkali. Lactose is fermented as frequently as are some of the other sugars. This sugar is acted on pretty constantly by *B. diphtheroides-liquefaciens* and *B. enzymicus*. It is rarely acted on by the other sub-groups. When a coagulum is formed it is usually due to the production of acid, although the experiments of Fox (1915 b) and others indicate that certain strains have a coagulating enzyme. In many instances the amount of acid formed was not great enough to coagulate the milk unless it was boiled.

Alternation between red and blue was observed not infrequently in some of my strains which were feeble acid producers. This may perhaps be due to the possibility that there is a small percent of glucose in the milk which undergoes fermentation, the resulting acid entering into combination with some neutral or buffer substance in the milk, only to be thrown out again as the resultant alkaline fermentation displaced it. With two of my strains the litmus cleared and became of a bordeaux-red color.

Potato

The same variation which characterizes the appearance of the various races on other media holds true for this one. In general it may be said that the more luxuriant growers are adapted to this medium. Pigment is readily produced on potato. The growth is generally very moist or slimy and not infrequently is of a dirty grey color. The majority of Fox's (1915 b) strains show no growth on this medium and the same can be said of mine. However when used in the proportion in which it is found in Bordet's medium potato seems to exert a favorable influence on all forms. Particularly has this been true in the isolation of *B. Hodgkini*, but this form grows as well if not better on glucose-blood-agar.

MUTATION

Roux and Yersin and other of the earlier investigators claim to have been able to produce a diphtheroid from a Klebs-Loeffler bacillus by growing it at high temperature. Hewlett and Knight (Park and Williams 1910) Richmond and Salter (1898) claim to have transformed *B. Hoffmanii* into the Klebs-Loeffler bacillus; the former by culture and passage through guinea-pigs and the latter by passage through gold finches. Bergey (1904), Williams and others have not been able to obtain these results. Fox (1915 a) attempted mutation by culture on different lymph gland media but was unsuccessful.

THERMAL DEATH POINT DETERMINATIONS

Twenty-four hour cultures of salt suspensions of the bacilli were filtered through cotton-glass-wool in order to remove the clumps. The homogeneous suspension was then drawn into thin walled capillary tubes 2 mm. in diameter by 8 cm. long. These were placed in the water bath at varying temperatures for varying lengths of time and then cultured for one week in glucose-serum-broth. The results are indicated in tabular form.

TIME OF EXPOSURE	TEMPERATURE	STRAIN	CULTURAL RESULTS AT			
			55°C.	60°	65°	70°
minutes	°C.					
10	55°	T-337	+	–	–	–
5	60°	x-320	+	–	–	–
3	65°	x-323	+++	–	–	–
2	70°	1	+	–	–	–

It will be seen that 60°C. was fatal to various strains of this group under the conditions of the experiment. The antiformin-fast strain x-323 was not affected by ten minutes exposure at 55 while the growth of the others was markedly inhibited.

ANTIFORMIN TESTS

Salt suspensions of the various strains after being filtered through cotton-glass-wool were mixed with equal parts of antiformin and placed at 37.5°C. for two hours. They were then washed with NaCl twice, centrifuged in NaCl and the sediment stained by Gram and cultured on blood-agar. The results are shown in table 2. There seems to be the same variability regarding this test that is displayed by the group in other respects.

The *B. Hodgkini* strains seemed to yield more of the Gram-positive organisms in the sediment than did the other races which showed a tendency to be resistant to antiformin. None of them could be cultured following this treatment. It would not seem that antiformin resistant organisms are limited to any one subgroup of the diphtheroids, although this resistance becomes progressively less as we approach the saprophytic members. Hall and Stone (1916) report their *B. flavidus* as non-resistant to the reagent and Fox's (1915 b) observations tend to show that the reaction is non-specific.

OXYGEN

Most of the races are facultative anaerobes, as may be inferred from the cultural results on gelatin stabs. A few instances of

strict anaerobosis have been reported. Dick (1915) describes a Gram-negative and a Gram-positive strict anaerobe isolated from the urine of cases of chronic non-suppurative nephritis. Torrey (1916) has isolated an anaerobe from the lymph glands of Hodgkin's disease which he has called *B. lymphophilus*. Bloomfield tentatively makes a "partial pressure" group of diphtheroids. He says that they are slow growing and rather anaerobic. Rosenow has often noticed this quality in both diphtheroids and streptococci. Strain 2 of my series was isolated from the blood and urine as a strict anaerobe. Voigt has isolated strict anaerobes from the vagina, (personal communication).

SUMMARY OF MORPHOLOGY AND CULTURAL CHARACTERS

The diphtheroids readily adapt themselves to artificial media, this being more noteworthy in the subgroups *B. diphtheroides-liquefaciens*, *B. Hoagii*, and *B. Hoffmanii*. The more fastidious strains usually require the presence of serum in the medium. Viability is prolonged on all media, especially gelatin. The same strains may contain variants giving rise to different types of colonies which increases the difficulties of isolation. Blood-agar is a favorable medium for separating mixed cultures. In general, both cultural and morphological characteristics can be correlated with the sugar and complement-fixation tests.

FERMENTATION

Sugar fermentation has been regarded for a long time as one of the best criteria for the separation of bacteria into classes. On reviewing the literature on this subject I was astounded at the number of apparently contradictory results obtained. Further study regarding the large number of factors involved in this reaction, some of which are beyond control, together with the variety of methods used, caused me to accept most gratefully any uniformity that might be discovered.

Before citing the results of the various observers in this regard, a discussion of some of the difficulties regarding fermentative

reactions in this group might be in order. When it was first discovered that the Klebs-Loeffler bacillus produced acid in glucose broth and *B. Hoffmanii* did not, a distinction between the two organisms was based on this point. Gradually it was found that there existed other diphtheria-like organisms which fermented glucose but which were non-toxic. Accordingly, for a long time various observers described different strains of diphtheroids, making their observations only on glucose and attaching a sonorous name to the organism, which was based very often on some minor characteristics, most commonly, the morphological appearance. The small number of sugars used was the first difficulty in the way of a successful grouping of different strains.

When more sugars were employed, each observer had his own method of determining when fermentation had taken place. Even to the present time a variety of methods is used, many of them being very questionable for a procedure of this kind. Few observations have been quantitative. Litmus has been freely used as an indicator both in liquid media like Hiss serum water and in solid media as well. With scantily growing organisms like some of the diphtheroids it is very difficult to ascertain whether growth has actually taken place in the presence of litmus. It is also subject to reduction decolorization changes which are not infrequent in this group. But its greatest fault, particularly when used with the diphtheroids is its lack of delicacy. Generally speaking these organisms do not violently attack sugars, and many reactions which are positive to phenolphthalein are lost with litmus.

For example: Fox (1915 b) in studies as late as 1915 reports that "observations on carbohydrates were made on litmus-agar-sugar mediums of reaction neutral to phenolphthalein." In this case the organism would be compelled to produce at least 1.5 percent acid before litmus would register it, a neutral phenolphthalein reaction being alkaline to litmus to the extent of 1.5 to 2.5 per cent. Since on many sugars, a variety of strains will not produce this amount of acid it is very easy to see how negative results might be obtained. Hiss serum-water with litmus as an

TABLE 3

Fermentation reactions

STRAIN NO.	SUCROSE	GLYCERIN	MANNITE	GLUCOSE	DEXTRIN	LACTOSE	MALTOSE	INULIN	SALICIN	RAFFINOSE	GAS	GROWTH
Sub-group B. enzymicus												
1	0.5	1.5	0.0	2.1	0.5	1.3	1.1	0.5	0.4	0.0	0	+++
2	0.5	3.4	2.7	2.8	0.5	0.4	0.7	0.5	0.0	0.4	0	+++
3	0.5	0.4	0.9	1.9	0.4	0.4	0.4	0.4	0.4	0.0	0	+++
9	0.9	0.3	0.4	0.7	0.3	0.4	0.3	1.2	0.0	0.0	0	++
11	0.6	0.3	0.45	0.9	0.4	0.4	0.5	0.8	0.5	0.6	0	++
13	0.0	0.65	0.0	1.5	0.4	0.45	2.3	0.3	0.3	0.3	0	+++
14	0.7	1.6	0.4	1.5	1.0	0.3	1.0	0.0	0.6	0.6	0	++
16	0.9	1.1	0.0	1.7	1.2	1.0	0.9	0.0	1.4	0.0	0	+++
33	0.4	0.0	0.6	0.8	0.7	0.4	0.4	0.3	0.0	0.0	0	++
x-323, 37	1.4	1.5	1.6	1.8	1.0	1.8	1.6	0.4	−0.4	0.9	0	+++
x-324, 38	1.2	1.2	1.4	1.5	0.9	1.2	1.2	−0.5	−0.4	0.0	0	+++
x-325, 39	1.6	2.5	2.2	2.8	1.5	1.4	1.1	0.0	0.0	0.0	0	+++
x-322, 40	2.6	2.8	2.5	2.8	1.8	2.6	2.1	1.1	1.1	1.0	0	+++
41	2.5	0.7	0.0	0.7	1.0	2.1	0.6	0.0	2.3	1.0	0	+++
42	1.7	0.8	0.0	2.0	0.5	2.7	1.2	0.0	0.0	2.0	0	++
x-319, 45	1.0	0.8	0.6	1.2	1.3	1.1	0.9	0.6	0.7	1.1	0	+++
Sub-group B. flavidus												
T-337, 35	0.0	0.6	−0.3	1.3	−0.4	−0.5	1.6	−0.6	0.0	−0.4	0	+++
Sub-group B. Hoagii												
4	0.5	0.0	0.0	0.5	0.0	0.0	0.0	0.9	0.0	0.0	0	++
5	0.4	0.0	0.0	0.3	0.0	0.0	0.0	0.4	0.0	0.0	0	++
6	0.8	−0.35	0.0	1.3	0.0	0.0	1.0	0.0	0.6	−0.3	0	+++
7	1.4	0.0	0.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0	+++
8	1.5	0.0	0.0	2.0	0.0	0.0	0.0	0.0	0.3	0.0	0	+++
19	2.0	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.3	0.0	0	+++
32	0.0	0.0	0.0	0.6	0.4	0.0	2.0	0.4	0.0	0.0	0	++
34	1.8	0.0	0.0	1.0	1.0	0.0	1.2	−0.5	1.0	0.0	0	+++
x-320, 43	0.0	−0.2	−0.3	0.7	0.0	−0.4	0.0	−0.4	0.0	0.0	0	+++
Sub-group B. liquefaciens												
21	−0.4	−0.3	−0.4	+1.1	−0.5	+0.4	0.0	−0.3	0.0	−0.3	0	+++
Sub-group B. Hoffmanii												
10	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
24	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
25	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
26	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
31	−0.5	−0.8	−0.7	−0.8	−0.4	−1.0	0.0	−0.6	0.0	−0.4	0	+++
x-321, 44	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0	++
Sub-group B. Ruedigeri												
Sub-group B. xerosis												
12	0.0	0.0	0.6	0.7	0.4	0.0	0.0	0.0	0.0	0.0	0	++

indicator is often used but its disadvantages can be easily seen, and it should be displaced by more accurate methods. If one wishes to work with a medium of this sort, by far the most delicate and satisfactory is the one devised by Holman (1914) for streptococcic work. Andrade's reagent (basic fuchsin) which is the indicator used in this medium has the advantage of being very sensitive to the presence of acid. It is rather tedious to prepare however. Other observers have used neutral red in solid media which is also more delicate than litmus (Teoumin, 1913).

A third source of diversity is found in the length of time the cultures remain in the incubator. Variation in this regard has ranged from two or three days to an equal number of weeks. With a frankly alkaline or frankly acid producing organism this factor would not be of much moment, but with a group that at one time may produce acid and at another alkali in the selfsame medium, it is obvious that it becomes an important factor for control if any uniformity is to be reached. Morse (1912 a) in her biometrical study of the diphtheroids has done more to correct this error than anyone else. She experiments with various strains on various sugars and finds that the sugars have a "time constant" for the production of maximum acidity which is different for every sugar. After this acid limit is reached, the organism attacks the protein of the medium and an alkaline reaction results. This has been shown by Kendall (1911) and Theobald Smith.

It should also be mentioned that adequate care must be taken to be sure that growth has occurred, as in its absence one would not expect fermentation. With doubtful strains this becomes a laborious matter in turbid or colored media. Chemically pure sugars should also be obtained in order to avoid another possible source of error. It is also important that sugars be fractionally sterilized as many of them are broken down by autoclaving.

It seems unnecessary to mention that the cultures tested should be pure. I have obtained a number of cultures from other sources which were free from organisms other than diph-

theroids but which contained more than one type of diphtheroid as was evidenced by the different sugar fermentations of the isolated strains.

Again it seems probable that certain races may at times lose their power of attacking certain sugars. I do not believe that this is a common occurrence especially outside of the body, but we have evidence that it does occur. J. H. Brown (personal communication) has definitely proved that such changes take place among the dysentery strains, and we have reasonable evidence that an organism may lose its power to ferment one sugar or acquire it for a sugar which it did not have, and still retain its other characteristics. *B. xerosis* is usually described as fermenting sucrose, yet I have a strain (number 12) typical in every other way and which on repeated tests did not ferment sucrose. Again Knapp (1904) finds a positive reaction to mannite while Zinsser is unable to verify the fermentation of that particular carbohydrate. Much more striking is the fact that *B. diphtheriae* is described as never fermenting sucrose, yet Graham-Smith (1908) has described a whole epidemic from which he obtained a sucrose fermenting true Klebs-Loeffler bacillus. The variability of the Klebs-Loeffler bacillus in this respect is well established.

Without doubt the best classification of the diphtheroids on the basis of sugar fermentations is the "Study of the diphtheria group of organisms by the biometrical method" by Morse (1912 a) As noted above she separated the diphtheroids into four main subgroups as follows:

(a) Group A is *B. Hoagii* which she names after Hoag who first described thoroughly a great many strains of this organism. She says that it ferments glucose and sucrose but not maltose. Regarding this last sugar she does not agree with Hoag since in his original article he states definitely that maltose is fermented and his tabular results bear out the statement. However this does not change the grouping. This organism was been described by more observers than any other of the diphtheroids except possibly *B. Hoffmanii.* About half of my *B. Hoagii* strains ferment maltose while the other half do not. On this basis it

will be very easy to place the so called *B. paralyticans* described by Ford-Robertson, (Morse, 1912 a). It ferments glucose, maltose and sucrose, and Morse is unable to place it on account of its maltose fermenting properties. It falls very nicely into one *B. Hoagii* subgroup. It can be very properly compared to the Klebs-Loeffler bacillus in its relation to lactose, and brings out a point which I wish to emphasize viz., that the fermentative history of this whole group shows that no hard and fast lines can be laid down, but that all the characteristics of an organism must condition its allocation.

(b) Group B is the second class of Morse' diphtheroids which she has named *B. flavidus*. Glucose is always fermented, maltose and glycerine usually but not sucrose.

(c) Group C or *B xerosis* always acidifies glucose and usually both maltose and sucrose.

(d) *B. Hoffmanii* is a nonfermenter.

Fox (1915 b) has studied a large number of strains from Hodgkin's glands as well as lymph glands from other conditions, particularly those draining enlarged joints. He tested the different strains on glucose, lactose, sucrose, maltose, mannite, glycerin, dextrin and galactose, but was unable to discover any striking regularities by this method. He did not work quantitatively and the other conditions which I have discussed on page 291 may contribute somewhat to the irregularity. However one seeming generality is noticed. The organisms isolated from the glands draining diseased joints have as a class a wider range of fermentation than the other strains. The possible significance of this fact I shall discuss later.

Hine (1913) attempts to classify the urethral diphtheroids by means of sugar reactions. He uses glucose, sucrose, maltose and lactose. Litmus is used as an indicator and seven days is the incubation period. The first class ferments no sugars (*B. Hoffmanii*); the second ferments glucose, sucrose and often maltose (*B. Hoagii*) and a third which he calls *B. coryza-segmentosus* ferments glucose, or glucose and sucrose. The urethral group proper ferments all sugars but lactose. These do not correspond to any of the other classes.

Torrey (1916) states that since no classification of the diphtheroids exists, he is not able to place accurately the various strains which he has studied, but forms three arbitrary groups for them based partly on sugar fermentation and partly on morphology. The vast majority of his strains fall in two common groups viz., *B. Hoffmanii* and *B. Hoagii*. From his brief description of some of his miscellaneous strains they resemble closely the *B. xerosis* or *B. flavidus* subgroup.

Teoumin (1913) studies the sugar reactions of 20 strains of pseudo-diphtheria. He has placed them in four classes yet they all have the same fermentative reactions, namely positive glucose and maltose. It is very evident that these groups correspond to Morse's *B. flavidus* and are identical with Hamilton's Group II.

DeWitt (1912) describes a pathogenic strain closely resembling *B. proteus* and producing both acid and gas in broth. Hamilton (1907) has also described several gas producing strains, and has studied 57 strains which fall into two groups. Her Group I had 40 representatives and fermented glucose and sucrose but was negative to maltose, lactose, and dextrin. This corresponds to Morse's *B. Hoagii*. ~~Group II~~ ferments glucose and maltose ~~but never sucrose~~. Dextrin is 60 per cent positive and lactose 10 per cent. This corresponds to Morse's *B. flavidus*. Group III is the Ruediger bacillus which produces a soluble toxin but ferments none of the sugars.

It would seem that the following sugar fermentation method advocated by Theobald Smith is the best one which we have at present; and I have used it in my work. Broth rendered sugar free by previous treatment with *B. coli* is given a reaction of plus 0.8 to plus 1 to phenolphthalein. Each tube contains 9 cc. One cubic centimeter of a 10 per cent solution of the various sterile sugars is then added to each tube with a sterile pipette, and incubated for forty-eight hours to insure freedom from contamination. The sugars are titrated on the eighth day, as at this time, a maximum acidity is produced in maltose, mannite, dextrin, lactose, raffinose, salicin and inulin. If no acid is formed with glucose, sucrose or glycerin at this time, the

latter is again titrated at the sixteenth day and the former sugars at the twelfth or thirteenth day. This latter procedure was adopted following the suggestion of the Morse article. A control tube was always incubated when the reaction determined on the sugars was titrated, since it was found by actual experiment that a variation of between 0.3 and 0.4 per cent might be produced as the result of evaporation of the medium with concentration of the acid it contained.

Although this method is the most accurate and serviceable of any in use at the present time, it has some faults which it might be well to point out and which stand in a fair way to be corrected in the near future by the application of more accurate physicochemical methods. It has been known for some time that the titratable acidity of a medium is not its actual acidity but only forms a relative and often an inaccurate guide to an estimation of the actual concentration of H-ions contained therein. This idea has been worked out and elaborated very convincingly by Clark (1915). To begin with, he shows that the titrametric method of arriving at the reaction of a medium is inadequate as at present used. He points out that the only correct means of arriving at its true reaction is by an estimation of the H-ion concentration. All nutrient media contain in varying proportion the so called "buffer substances" of which peptone and phosphates are two familiar examples. These substances are able within limits to absorb acid as it is produced by an organism. The organism is thus protected from its action and is enabled to produce further acid. When titration is performed the free H-ions are neutralized and then dissociation of the buffer substance takes place yielding more H-ions. In this way the same organism might produce a much larger amount of titratable acid on one medium than on another. This is one way in which to account for the very large amount of acid produced by a number of Morse's strains.

Clark also criticizes severely the titration of media while hot. He claims that the enormous amount of dissociation resulting causes a variable reading.

W. T. Bovie (1913) has recently devised an ingenious method

for measuring the H-ion concentration. It will still be some time however before one is able to make such determinations with facility, but it indicates a great advance toward the solution of a problem which has given much trouble to all who have wished for a really accurate notion of the reaction of bacterial culture media.

I have used ten fermentable substances with the various strains in my series. The monosaccharide glucose; the disaccharides, sucrose, maltose and lactose; the higher saccharides, raffinose, dextrin, inulin and salicin; and the alcohols, glycerin and mannite. The results in the appended table 3 represent the percentage amounts of normal NaOH required to neutralize the acid developed by the organism. Phenolphthalein was used as an indicator. A minus sign preceding a number indicates alkali production. A special column for growth is shown which shows that any negative results were not due to the fact that insufficient growth was obtained. Plus three indicates a luxuriant growth plus two, moderate; and plus one a scant growth.

ANALYSIS

From a survey of the sugar reactions, it is seen at the outset ~~that it will be neither possible nor necessary to~~ exclude from a subgroup an organism which does not ferment precisely the same substances as the original strain after which the subgroup was christened. However desirable such a clean cut grouping might be, there is no criterion of classification among these organisms which will be rigidly constant. I have already indicated a great many reasons why this must be so, and have substantiated my contention with evidence collected from the literature.

None of my strains were aerogenic. Some strains produced no acid on any of the sugars, while others had a wide fermentative range. Between these extremes one finds all gradations. Quantitatively the diphtheroid group does not produce a high percentage of acid, although some of Morse's strains compare favorably

with the amount produced by the streptococci. Torrey (1916) also describes an anaerobe producing a very high amount of acid.

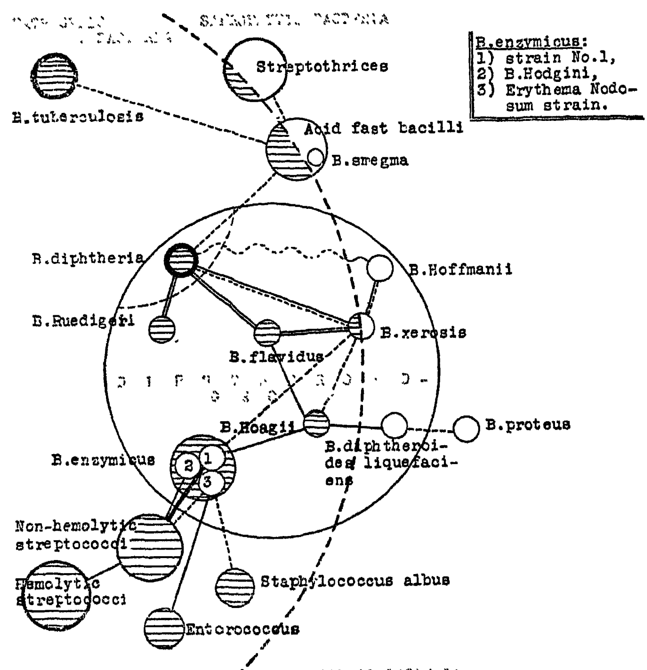
Six of the strains were *B. Hoffmanii*, 10, 24, 25, 31, 43, and 44, producing no acid from any sugar, but often yielding an alkaline reaction. Strains 4, 5, 7, 8, 19, 34, acidified glucose and sucrose constantly and in some cases salicin or inulin. Strain 34 also fermented maltose. These strains correspond to *B. Hoagii* of Morse. One strain, 12, fermented glucose and dextrin and was obtained from Dr. Wolbach's laboratory as *B. xerosis*. This organism usually ferments sucrose also but did not do so in this case. It was typical in all other respects. Strain 32 might be considered a variant of *B. Hoagii*. Strain 35 fermenting maltose and glucose was typical in all respects of Morse's *B. flavidus*.

Strains 1, 2, 3, 9, 11, 13, 14, 16, 33, 37, 38, 39, 41, 42, and 45, although isolated from a variety of sources, I have placed in one group which I have called *B. enzymicus* on account of the wide range of sugar fermentation which they possess. It is certain that these organisms fall into a group which is different in many ways from the other groups. They ferment from six to ten sugars which is a phenomenon that has been commented on only once before to my knowledge. Rosenow (1915) mentions that his erythema nodosum strains have a wide range of fermentation although he does not say how many sugars are fermented.

It is possible that this group may have to be further subdivided at a later time. Strains 37, 38, 39, and 40 were given me by Dr. Bunting and were isolated from Hodgkin's glands and in addition were strains with which he had produced results in animals. It is noteworthy that qualitatively and quantitatively they are practically identical. Strain 3 is a strain of my own isolated from Hodgkin's glands which has nearly the same reactions as Bunting's strains. The remaining characteristics of this subgroup have been discussed in Part I, Journal of Bacteriology, March, 1917. Strain 45 (one of Bunting's strains from pseudoleukemia) ferments all ten sugars and is thus fairly closely related to the Hodkgin's strains in that it

is a powerful fermenter. However the Hodgkin's strains do not ferment inulin and salicin although strain 37 attacks raffinose slightly. The chronic leukemia strain number 44 and the Banti strain number 43 ferment none of the sugars, and on this basis must fall into the *B. Hoffmanii* class or the *B. Ruedigeri* class. The latter however has a soluble toxin. These strains are both from Dr. Bunting.

CHART NUMBER 1.



In Chart I, I have indicated diagrammatically the interrelationships of the various subgroups and their connection with the Klebs-Loeffler bacillus, as well as the relation of the entire diphtheria group to other micro-organisms. The broken curved line bounds a segment of a circle which represents the

domain of the pathogenic forms. Since there can be no hard and fast line separating the pathogenic and saprophytic fields, the division is pictured by a broken line. The larger area surrounding the arc of this circle represents the domain of the saprophytic organisms. The entire diphtheria group is shown by the medium sized circle occupying the border line position, part lying in the saprophytic field and part in the pathogenic.

The individual subgroups of diphtheroids are shown by small circles, and are shaded or not according as they fall within or without the pathogenic field. A double solid line connecting the subgroups indicates a close relationship; a single solid line indicates a moderate linking and a dotted line a distant relationship. A curved line indicates a mutation.

Beginning at the small area indicating the *B. diphtheriae* there is shown a close direct relationship between it and *B. flavidus*. If there is any organism which could properly be called an avirulent diphtheria bacillus this is certainly the one. I believe that this designation should be applied to an organism similar in most respects to the Klebs-Loeffler bacillus but lacking the specific toxin formation of the latter. In the dry growth on blood and serum media, the firmness of the colonies and their adherence to the media in old cultures, *B. flavidus* is much more like the diphtheria bacillus than are most of the diphtheroids. It also forms a very distinct, friable pellicle on glucose-broth which falls to the bottom on slight agitation of the tube. Involution forms appear in the older cultures. It ferments glucose, maltose, and glycerin, but not sucrose. It is pathogenic for guinea-pigs but does not produce the lesions of the diphtheria bacillus. Most noteworthy was the evidence of complement fixation tests, (see Part III). The fact that this strain was practically alone in its separation from the other strains was noticeable. It is quite closely related to *B. xerosis* (strain 12), a fact that Morse's complement fixation work has also brought out. Hamilton (1907 b) also believes this glucose-maltose fermenter to be closely related to the true diphtheria bacillus. Four of the seven strains which she studied killed guinea-pigs. She describes it as Group II of her classification of the diphther-

oids, but refers to it as *B. xerosis*. Morse has identified Hamilton's Group II as *B. flavidus* of her own classification. The strain which I have studied is one that Teacher (1915) has shown to be the causative organism in a epizoötic of infective abortion in guinea-pigs. To reiterate briefly: One finds the cultural, morphological, immunological and sugar reactions of *B. flavidus* to be strikingly similar to those of the true diphtheria bacillus, but inasmuch as it produces no specific toxin it must be classed among the diphtheroids.

Closely related to the diphtheria bacillus as well as to *B. flavidus*, is *B. xerosis*. It also has a scant, dry, adherent growth and a diphtheria-like morphology. It is described as characteristically a sucrose fermenter, although the strain which I have studied has no action on this sugar. Its complement-fixation and agglutinin reactions show that it is closely related to *B. flavidus*. It has only been described by one observer as having been pathogenic for guinea-pigs (Eyre, 1896), producing only local edema, decided loss of appetite and weight. Escherich and C. Frankel (1896) and F. Schanz (1896) have all noted the strong resemblance between the avirulent Klebs-Loeffler bacillus and *B. xerosis*. The only reference to its pathogenicity in man is the report of Eyre (1896) who isolated it from 15 cases of chalzion, and reproduced the condition in the eyes of animals. Accordingly I have given it a very close relationship to both the Klebs-Loeffler bacillus and *B. flavidus*.

B. Hoagii is an entirely different type of organism from the previous two described. It is a luxuriant grower on almost any kind of media, under almost any conditions, and has the widest distribution of any of the subgroups. Hoag describes it as fermenting glucose, sucrose, and maltose. He has isolated 150 strains of it. The so called *B. paralyticans*, supposed by Ford-Robertson and others to have an etiological relationship to paresis is merely the saprophytic *B. Hoagii*. It has the same sugar reactions. Hamilton (1907) has also studied 40 strains of this organism wrongly calling it the psuedo-diphtheria bacillus. She also finds that it ferments only glucose and sucrose.

My strains 4, 5, 19, and 34 were representatives of this sub-

group. Strains 4 and 5 fermented glucose, sucrose and inulin, while 19 fermented salicin in place of inulin; strain 34 in addition to glucose, sucrose and maltose fermented salicin and dextrin. A study of the characteristics of these organisms shows that they all belong to the same group, despite the fact that the fermentation varies somewhat. They have in common the fermentation of glucose and sucrose. In this connection, I wish to mention strain 32 which has the same fermentative powers as strain 34 except for a doubtful reaction with sucrose. Even glucose is fermented only to 0.6 per cent. In addition it liquefies serum agar (Loeffler's). In spite of these differences I consider it to be merely a variant of *B. Hoagii* and not deserving of some special name. Recourse to the immunological reactions of the two strains seems to bear out the contention. *B. Hoagii* is also related to strains 1, 2, 3, and x-323 which subgroup is located at the other extreme. For these reasons I regard this subgroup as occupying an intermediate position between *B. flavidus* on one hand and *B. enzymicus* on the other. When pathogenic, it is usually found in connection with suppurations, usually not initiating a pathologic process, but sustaining it when once started by a more virulent organism, Hamilton (1907), Bergey (1904). Experimentally I have produced local subcutaneous abscesses in guinea-pigs with *B. Hoagii* after the animal had previously received an intraperitoneal injection of the same organism.

I have next to consider an organism which I believe merits a distinctive position. Ruediger (1903) and later Hamilton (1904) have described a very important bacillus, which morphologically resembles the diphtheria bacillus, but culturally and fermentatively seems more closely allied to *B. Hoffmanii*. It grows heavily on agar, and gives diffuse cloudiness in broth. Its most noteworthy cultural characteristic is its decolorization of litmus. This is probably due to its reducing action which is possessed to a greater or less degree by most members of the group, although complete decolorization of litmus is not usual especially in the absence of acid formation. This form ferments no sugars but produces a soluble toxin which is very pathogenic

for guinea-pigs. The toxin is not neutralized by anti-diphtheritic serum. I was not able to obtain a culture of this organism but Hamilton's immunological work indicates that it is distinct from *B. Hoagii* and *B. flavidus.* I have given it a separate place closely related to the diphtheria bacillus, and more distantly related to *B. Hoffmanii* and have named the subgroup *B. Ruedigeri* after the author of the original description of this organism.

B. Hoffmanii, I have considered as a mutant in accordance with the opinion expressed by Flexner and others. Its total fixing value is only eight, showing that the cross-reactions with the other sub-groups are not great. It has slight cross-fixation with *B. enzymicus.* Kolmer (1916) has shown that there is a definite cross reaction between this organism and *B. diphtheriae.* Strains 10, 24, 25, 31, 43, and 44 of this series belong in this group.

A very interesting bacillus was described by Graham-Smith (1904) in 1904 which he has called the *B. diphtheroides-liquefaciens.* He first isolated it from the mouth of a patient supposed to be suffering from diphtheria. It is characterized by the following features. The organisms are very long and curved and may be motile. There is practically no segmentation or involution produced; slow liquefaction of serum and gelatin; slow but abundant growth on potato. Glucose, or glucose and lactose are fermented and milk usually coagulated. Litmus is decolorized. Nitrates are reduced and much indol is formed. No gas is produced.

Hamilton (1904) has described an organism which can be placed in this subgroup but which produces gas in glucose broth. Klein (1903) has described a *B. diphtheroides* which also liquefies both serum and gelatin, produces hepatization in rat's lungs and is pathogenic for guinea pigs. De Witt's (1912) proteus-like organism may also be included in this group. Strain 21 of my series must also be placed here. It liquefies gelatin but not serum, ferments glucose and lactose and coagulates milk with decolorization of the litmus. This assemblage of organisms is very closely related to *B. Hoagii* (strain 34) but particularly to strain 32 which is a variant of strain 34. These sub-

groups have a distinct relationship to the saprophyte *B. proteus* a fact which I have shown in the diagram.

I now come to a subgroup which I have designated *B. enzymicus* on account of the range of its sugar fermentation. Its distinctive characteristics can be learned in detail by a study of strain 1 which I take to be a representative member of the subgroup.

It has three main characters not common to the other subgroups: first, a remarkable pleomorphism; second, a wide range of sugar fermentation; and third, a distinct biological relation to the streptococci.

On account of these qualifications I have represented this subgroup as being more intimately connected with the non-hemolytic streptococci than with the other members of the diphtheroid group. This particular strain produces in addition a poisonous substance in the broth filtrate which resembles in many ways a true toxin. Strain 1 from a purely morphological standpoint can be called either a diphtheroid bacillus or a non-hemolytic streptococcus. It can be made to grow in long chains of diplo-streptococci. Biologically it has the features of a streptococcus of a moderate grade of virulence in that it constantly produces suppurative arthritis, cholescystitis, and myositis in animals. Reference to the immunological reactions shows how definite is its relation to the non-hemolytic members of this group. The viability of this strain is much more prolonged than that of the streptococcus and its virulence is also lost much sooner on artificial media. It then occupies an intermediate position between the remainder of the diphtheroid group and the streptococci.

Rosenow's erythema nodosum strain appears to show almost identical cultural and morphological features, as well as pathological, although he has not attempted to demonstrate its relation to the streptococci. However he has suggested it. It can be placed with certainty in the subgroup *B. enzymicus*. Likewise, I believe that the so called *B. Hodgkini* can be placed in this subgroup, although if it is demonstrated to have the etiologic relation to Hodgkin's disease that is now claimed, it will no

doubt merit a more specific ranking. The strains in this series which I have studied and placed in this group are strains 1, 2, 3, 9, 11, 13, 14, 16, 33, 37 (x-323), 38, 39, 41, 42. Without exception the strains in the bacillary form were very scant growers, even on blood or serum media. I have been able permanently to alter the morphology of most of them, and in every case to develop quickly a very luxuriantly growing strain from one cultivated with difficulty.

I shall later describe the relation of this group to the enterococcus of Thiercelin and the complement-fixation tests show some cross-reaction with the staphylococcus. This work in connection with that of Frankel and Much who interpret *B. Hodgkini* as a special form of the tubercle bacillus, is evidence that the group touches on the more definitely acid-fast organisms such as the *B. tuberculosis.* Gordon (Graham-Smith 1908) has shown the relation between certain streptothrices and the diphtheria group. As a result of this evidence, I have placed as contiguous, the *B. tuberculosis* and the streptothrices, as well as the enterococcus, the streptococcus, the staphylococcus and *B. proteus.*

Any of the diphtheroid subgroups under favorable conditions may become pathogenic, although this proclivity is much more marked in some than in others. *B. enzymicus* containing as it does examples of the erythema nodosum, streptococcoid, and *B. Hodgkini* strains would seem to represent an adaptable assemblage which can easily become pathogenic. Likewise we have many examples of the pathogenicity of *B. flavidus* and *B. Ruedigeri.* On the other hand it is very rare to find *B. Hoffmanii* pathogenic, although Fox (1915a) and others have reported it as the probable cause of certain benign unilateral types of pharyngitis.

I shall now consider the various diphtheroids which have been reported from time to time under various names and relate them to this classification so far as possible. Cautley (1894) described an organism which he calls *B. coryza-segmentosus* which he believed to be the cause of some of the common colds. Gordon (1901), Benham (1906) and Graham-Smith (1908) have

isolated various strains of it. It ferments glucose, galactose, lactose, levulose, sucrose and maltose. Benham has renamed it *B. septus*, on account of its morphology at the particular time when he happened to observe it. It corresponds well to strain 16 of my series isolated from the nasal discharge of a fresh cold, and strain 11 isolated from a normal throat. It falls under *B. enzymicus* or the group of active fermenters. We know that it is not related to coryza except as a secondary invader. Neumann (1902) from a study of 206 cases of nasal colds, believes that diphtheroids have no etiological relation to the condition. He found them in 98 per cent of diseased noses, and 100 percent of normal noses.

Graham-Smith (1908) has isolated a diphtheroid which he calls *B. maculatus*. Its poor growth on most media is its most noteworthy characteristic. It ferments glucose. From this general description it probably falls into the *B. xerosis* or *B. flavidus* subgroup. He says that it resembles closely a non-virulent diphtheria bacillus, in which case it would naturally fall in the latter group. I see no reason for giving it a separate name. *B. muris* described by Klein (1903) is an interesting organism. It was isolated from the hepatized lung of a white rat, and he was able to reproduce this lesion in other rats. Nothing but a large local abscess was produced in guinea-pigs. Culturally and morphologically this form has a strong resemblance to the true diphtheria bacillus. In the absence of a more complete description it could be placed in the *B. flavidus* subgroup. Bergey (1904) and Dean (Graham-Smith 1908) also isolated organisms from abscesses in a leprosy-like disease of rats which have the same characters as Klein's *B. muris*. MacFadyean and Hewlett (Graham-Smith 1908) have isolated the same bacillus from the mouth of healthy and diseased pigeons.

Nakanishi (1900) and Brown (Graham-Smith 1908) isolated an organism from cases of vaccinia and variola which they called *B. lymphae-variabilis*. It is easily identified as belonging to the subgroup *B. Hoffmanii*. Galli-Valerio (Graham-Smith 1908) has called his organism recovered from vaccine lymph, *Corynebacterium vaccinae*. This also is *B. Hoffmanii*. Klein

describes the *B. xerosis variolae*, and *B. albus variolae* from glycerin emulsions of small-pox crusts which are in all probability the ordinary *B. xerosis*. The descriptions are inadequate. De Simoni (Graham-Smith 1909) isolated an organism from the same sources which corresponds to *B. Hoagii*. Levy and Fickler (1900) isolated a *Corynebacterium lymphae-vaccinalis* which is very probably *B. flavidus*.

Graham-Smith's *B. xerosis canis* is merely the ordinary *B. xerosis*. His *B. diphtheriodes gallinarum* is very probably *B. flavidus* and his *B. ceruminous* is the common *B. Hoagii*. His *B. diphtheroides-brevis* and *citreus* also have nothing distinctive and can be grouped under *B. Hoagii*. Graham-Smith has also isolated a *B. auris* from the ear. De Simoni (Graham-Smith 1908) has found the same organism there, while Griffith has isolated it from the normal eye. Bergey has isolated an identical organism from the vagina which was pathogenic for guinea-pigs in large doses. All these organisms can be grouped under *B. Hoagii* although the sugar reactions are only given in part. Certainly there is nothing distinctive about any of them.

Bloomfield (1915) divides the diphtheroids of lymphatic glands into three arbitrary groups, although there is really no biological classification made. Group I he correlated with surface saprophytes, probably meaning *B. Hoagii*, *B. Hoffmanii* and *B. xerosis*. In Group II he describes a short pleomorphic bacillus mildly anaerobic, and another type resembling a coccus with the same characters as the short pleomorphic bacillus. They are probably identical, one being the coccus form of the other. It probably would be placed with *B. enzymicus*. Group III is heterogenous.

Hine (1913) has classified urethral organisms most of which he has identified with *B. coryza-segmentosus*. As I have already suggested, all these organisms can be grouped under *B. enzymicus*. His Group I is *B. Hoffmanii* and his Group II is *B. Hoagii*.

Torrey studied the lymph gland diphtheroids and placed them in three arbitrary groups. The majority of the strains which he places in Groups I and II can be identified with *B. Hoagii* and *B. Hoffmanii* from their action on sugars. Some

strains in class I belong to *B. enzymicus*. In class III *B. xerosis* or *flavidus* predominates. He describes a *B. lymphophilis* which I have not had opportunity to study. It is an anaerobe obtained mostly from Hodgkin's glands and ferments glucose, glycerin and sucrose. He believes it to be a distinct species.

The classification of Teoumin (1913) based on sugar reactions and agglutination tests I have already referred to on page 294. These organisms fermenting maltose, levulose and glucose plainly belong to the *B. flavidus* subgroup. Wolbach and Honeij (1914) describe a diphtheroid bacillus which they believe to be identical with the majority of those described from leprosy. It is a very typical example of *B. flavidus* in its morphological and cultural characteristics and in its sugar reactions.

De Witt (1912) has described a very unusual pathogenic diphtheroid organism which she thinks is closely related to *B. proteus*. It liquefies both serum and gelatin, is actively motile, produces gas in glucose broth, coagulates milk and develops indol. This form is closely related to *B. diphtheroides liquefaciens* of Graham-Smith and undoubtedly will fall in this subgroup.

(To be continued)

THE FUCHSIN-ALDEHYDE REACTION ON THE ENDO MEDIUM

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Endo (1904) suggested the use of a medium of the following composition for the differentiation of *B. typhosus* from *B. coli*-like organisms:

Neutralized nutrient agar (3 per cent agar)	1000 cc.
Chemically pure lactose	10 grams
Alcoholic solution of fuchsin	5 cc.
Ten per cent sodium sulfite solution	25 cc.
Ten per cent sodium carbonate solution	10 cc.

The medium had a light pink color when hot and was practically colorless when poured into Petri dishes and allowed to harden. On this medium Endo observed that *B. coli* and its close allies gave a distinct red coloration while *B. typhosus* was practically colorless. The red color of the *B. coli* colonies, according to this author, was due to the formation of acid but he did not give any experimental evidence to substantiate this belief.

Harding and Ostenberg (1912) have shown that the reaction is due to aldehyde formation. These authors suggest, also, that by the substitution of other carbohydrates in place of lactose in the Endo medium this reaction might be used as a means of differentiation of the colon-typhoid-dysentery group in place of acid production.

Robinson and Rettger (1916) have stated more recently that the red coloration of colon colonies on Endo medium is the result of acid production and not of aldehyde formation. These

[1] The author desires to acknowledge the valuable assistance of Dr. R. E. Buchanan and Prof. Max Levine in the preparation of this paper.

authors quote Grey as having found an aldehyde (acetaldehyde) produced in his fermentations only under anaërobic conditions.

It is the purpose of this report to explain, if possible, the discordant results by a comparative study of some of the reactions with acid and basic fuchsin which have been decolorized with sodium sulfite and bisulfite and to compare these reactions with those obtained when the true Schiff's fuchsin-aldehyde reagent[2] is employed.

REACTIONS WITH ACID AND BASIC FUCHSIN DECOLORIZED WITH SODIUM SULFITE

Saturated aqueous solutions of acid and basic fuchsin were decolorized with sodium sulfite. With these solutions qualitative tests with concentrated acids and 40 per cent formaldehyde were carried out. Four or five drops of the test substances were added to five to seven cubic centimeters of the reduced fuchsin solution. A pink color was taken as positive. The results are shown in table 1.

TABLE 1

Reactions of acids and formaldehyde with acid and basic fuchsin decolorized with sodium sulfite

	HCl	H_2SO_4	CH_3COOH	$CH_3CHOHCOOH$	HCHO	HCl AND HCHO
Acid fuchsin......	++	++	+++	+++	+++	+++
Basic fuchsin.....	−	−	++	+	−	+++

From table 1 it is to be seen that the acid fuchsin showed no marked differentiation between aldehyde and acid although the mineral acids did not show quite so strong a red color as was produced by the organic acids and formaldehyde. With the basic fuchsin the mineral acids produced a straw colored solution. The organic acids showed a slight reaction which might be produced by traces of formaldehyde as an impurity and, perhaps also, by the formation of a strong base and weak

[2] According to Mulliken Schiff's fuchsin-aldehyde reagent is prepared by dissolving 0.2 gram basic fuchsin in 10 cc. freshly saturated solution of sulfur dioxid. After standing for some hours the solution should be a straw color. This is then made up to 200 cc. with distilled water and kept in a dark place.

acid combination, e.g., sodium lactate, which will give a reaction with the reduced fuchsin. The reaction with formaldehyde was negative. With an acid also however, a very strong reaction was obtained. This showed that the coloration with formaldehyde is dependent upon an acid medium.

Endo plates were poured using basic fuchsin decolorized with sodium sulfite. A loop of different dilutions of hydrochloric, sulfuric, acetic and lactic acids was placed upon the medium and allowed to incubate at 37°C. for twenty-four hours. The twenty-fifth, fiftieth and one hundredth normal acids were used and in no case was a pink or red color produced. If, however, a loop of formaldehyde and a loop of fiftieth normal acid (any one of the four acids used), were added a very strong reaction was given. A loop of 40 per cent formaldehyde alone did not produce a red coloration upon the plate.

From the experiments outlined above, it is to be seen that acid fuchsin decolorized with sodium sulfite does not differentiate sharply between aldehyde and acids. The basic fuchsin shows a coloration with concentrated organic acids, but none with concentrated mineral acids. Formaldehyde alone does not give a reaction but when an acid is present a pronounced red color is produced.

REACTIONS WITH ACID AND BASIC FUCHSIN DECOLORIZED WITH SODIUM BISULFITE

Acid and basic fuchsin were decolorized with sodium bisulfite and qualitative tests with concentrated acids and 40 per cent formaldehyde were carried out as above. The results are shown in table 2.

TABLE 2

Reactions of acids and formaldehyde with acid and basic fuchsin decolorized with sodium bisulfite

	HCl	H_2SO_4	CH_3COOH	$CH_3CHOHCOOH$	HCHO	HCl AND HCHO
Acid fuchsin.....	++	++	+	+	+++	+++
Basic fuchsin....	−	−	+ (later decolorized)	+ (later decolorized)	+++	+++

The acid fuchsin as before did not differentiate sharply between acid and aldehyde. The organic acids did not produce quite so strong a color as the mineral acids. The basic fuchsin did not give a permanent red color with either mineral or organic acids. Formaldehyde gave a very strong coloration without the addition of acid because the bisulfite contains an acid radical which will serve to carry out the reaction. The results upon the Endo plates were the same as with the fuchsin reduced with sulfite solution. This is explained by the neutralization of the acid by the addition of the sodium carbonate solution in the Endo medium.

The most important point which the experiments with the sulfite and bisulfite show is that an acid radical is necessary for a strong red coloration when an aldehyde acts upon the reduced basic fuchsin.

REACTIONS WITH BASIC FUCHSIN DECOLORIZED WITH SULFUR DIOXID (SCHIFF'S FUCHSIN ALDEHYDE REAGENT)

With Schiff's reagent acids did not give any coloration, although acetic acid gave a slight pink color possibly due to traces of aldehyde. Strong caustics, potassium and sodium hydroxide gave a slight pink, due, as the chemists have shown, to the formation of a strong base and weak acid combination. Strong ammonium hydroxide did not give a pink color. Lactic acid neutralized with sodium carbonate gave a light pink.

Robinson and Rettger reported that concentrated lactic acid as well as dilute mineral acids gave a red color upon an Endo plate. It had been observed in this laboratory that the concentrated acid gave a red color with the formation of gas from the excess sulfite and carbonate, but no color was produced by any acid which approximated the concentration of the acid produced by *B. coli*. These statements applied only to decolorized basic fuchsin, however, as a strong color may be obtained with acid fuchsin when tested with either concentrated or dilute acids. It is possible, therefore, that these authors used acid fuchsin in their Endo medium; at least their results could be easily explained upon this hypothesis.

DO BACTERIA PRODUCE ALDEHYDE?

A number of organisms were grown in a medium of 0.5 per cent carbohydrate, 0.5 per cent peptone and 0.5 per cent dipotassium phosphate and were tested for aldehyde formation with Schiff's fuchsin aldehyde reagent. All cultures were incubated at 37°C. and tested in twenty-four hours and seventy-two hours. The results are given in table 3.

TABLE 3

Production of aldehyde in a medium containing 0.5 per cent each of dipotassium phosphate, peptone, and carbohydrate

	GLUCOSE		LACTOSE*	
	24 hours	72 hours	24 hours	72 hours
B. acidi-lactici 131	+	+	+	+
B. acidi-lactici 135	+	+	+	+
B. communior A. 123	+	+	−	+
B. communior A. 19	+	+	+	+
B. communior B. 144	+	+	+	+
B. communis B. 125	+	+	+	+
B. aerogenes	+	+	+	+
B. cloacae	+	+	+	+
B. pyocyaneus	−	−	−	−
B. pneumoneae	+	+	+	+
M. roseus	−	−	±	+
B. subtilis	+	+	−	−
B. prodigiosus	+	+	−	−
B. phosphorescens	−	−	−	−
B. typhosus (1)		+		−
B. typhosus (2)		+		−

* The lactose medium did not give a test for monosaccharides after sterilization.

It is evident from the above table that the colon-aerogenes group will produce aldehyde when grown in a sugar medium. Since the concentration of acid, produced by *B. coli*, upon the Endo plate is in itself not sufficient to produce a pink or red color the typical *B. coli* reaction must of necessity be due to the formation of both acid and aldehyde.

SUMMARY

Aldehyde formation by many bacteria may be demonstrated by Schiff's fuchsin-aldehyde reagent in glucose and lactose media.

Decolorized basic fuchsin reacts with aldehydes only in the presence of an acid to give the red color of the fuchsin-aldehyde test.

The red color of the *B. coli* group upon the Endo plate is due to the production of aldehyde together with acid.

Acid fuchsin which has been decolorized with sodium sulfite or bisulfite will give a red coloration with either acid or aldehyde. The color may be discharged by the addition of alkali.

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THE IMPORTANCE OF UNIFORM CULTURE MEDIA IN THE BACTERIOLOGICAL EXAMINATION OF DISINFECTANTS

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Neither of the two methods for the bacteriological standardization of disinfectants which are in common use today has proven entirely satisfactory. Even in the hands of the most careful workers wide discrepancies in results have occurred, both with the Hygienic Laboratory and Rideal-Walker tests. While it is probably true that a few individuals have succeeded in so controlling their work as to obtain generally uniform and consistent results, a method worthy of general adoption must be capable of yielding satisfactory results in the hands of any well trained bacteriologist. It is hoped that the present investigation will throw some light upon the fundamental principles involved and stimulate such further investigations as may be necessary for the development of a universally satisfactory procedure.

In the past the general tendency has been to lay the blame for discordant results upon the so-called "personal equation." In our experience the "personal equation," in so far as the actual manipulation of the test is concerned, does not enter into the problem at all. Any two workers, provided they are sufficiently experienced and dexterous with their hands to perform the necessary manipulations with accuracy and despatch, will obtain comparable results provided they are working with uniform materials throughout.

After watching the results of a large number of tests extending over a long period of time our attention was drawn to the fact that duplicate tests made with the same batch of culture

medium always gave uniform results, and that when discrepancies did occur, it was almost invariably when different batches of culture medium had been used for growing the test cultures or the subcultures. This led to the keeping of detailed records of all media used in this laboratory for the testing of disinfectants, including both the Rideal-Walker and Hygienic Laboratory broths. These records, which now cover a period of more than

TABLE 1

Variation in coefficient with different batches of media

Hygienic Laboratory method

DATE OF TEST	MEDIA FOR TEST CULTURES		SUB-CULTURE MEDIA, DATE MADE	COEFFICIENT	
	Date made	Amount of growth		Disinfectant A	Disinfectant C
1915	*1915*		*1915*		
July 2	June 28	Medium	July 1	15.00	5.25
July 3	June 28	Medium	July 1	15.25	5.50
July 5	June 28	Medium	July 2	15.00	5.47
July 6	June 28	Medium	July 2	15.25	5.47
July 7	June 28	Medium	July 2	15.06	5.50
July 8	July 2	Medium	July 6	15.12	5.37
July 10	July 2	Medium	July 10	15.50	5.25
July 12	July 3	Medium	July 10	15.00	5.50
July 13	July 10	Light	July 10	16.87	6.37
July 14	July 10	Light	July 10	16.62	6.41
July 15	July 10	Light	July 10	17.12	6.56
July 16	July 10	Very light	July 16	17.31	6.41
July 19	July 16	Medium	July 16	15.87	5.62
July 20	July 16	Medium	July 19	15.25	5.25
July 22	July 16	Medium	July 19	15.00	5.37
July 23	July 19	Light	July 20	17.12	6.67
July 24	July 19	Light	July 20	17.50	6.75
July 26	July 23	Medium	July 23	15.25	5.50

two years, include the following points: the serial number of each lot of media, the particular jar of meat extract and peptone used in its preparation, the batch of media used each day for growing the test cultures, the relative amount of growth upon test cultures, and the number of each lot of media used for subcultures. These records, which now include more than 1500 separate tests by both methods and more than 200 different

lots of culture media, have proven of the utmost value and have led to the firm conviction that a great majority of discordant results are due to variations in culture media. Never during this time has there been the slightest difficulty in checking our coefficients within very narrow limits when the same lot of culture medium was used. When on the other hand two or more lots of media were employed for check tests, wide variations have frequently occurred.

TABLE 2

Variation in coefficient with different batches of media

Rideal-Walker method

DATE OF TEST	MEDIA FOR TEST CULTURES		SUB-CULTURE MEDIA, DATE MADE	COEFFICIENT	
	Date made	Amount of growth		Disinfectant A	Disinfectant C
1915	*1915*		*1915*		
July 20	July 18	Heavy	July 19	18.00	6.50
July 21	July 19	Heavy	July 19	18.88	6.50
July 22	July 19	Heavy	July 19	18.00	6.25
July 24	July 19	Heavy	July 22	18.00	6.00
July 26	July 19	Heavy	July 22	18.00	6.00
July 27	July 22	Medium	July 22	20.00	7.50
July 29	July 22	Medium	July 22	21.10	7.00
July 30	July 22	Medium	July 29	19.90	7.50
August 2	July 22	Medium	July 29	20.00	7.25
August 3	July 29	Heavy	July 29	18.00	6.50
August 4	July 29	Heavy	August 3	18.00	6.50
August 6	July 29	Heavy	August 3	18.00	6.75
August 7	August 3	Medium	August 3	21.00	7.50
August 9	August 3	Medium	August 3	21.00	8.00
August 10	August 3	Medium	August 10	20.00	7.50
August 11	August 3	Medium	August 10	21.00	7.75
August 12	August 12	Heavy	August 12	18.88	6.50

Tables 1 and 2 show the record of a series of tests by both the Hygienic Laboratory and Rideal-Walker methods on each of two disinfectants. The tables speak for themselves and show clearly the variations which may be anticipated when different batches of media are used for check tests. Extreme care is used in the preparation, adjustment and sterilization of all media, and every precaution taken to have the procedure uniform in

every detail. Yet, in spite of the utmost care, different lots of media are at times sufficiently unlike to cause serious deviations in coefficients, this condition being evident with either the Hygienic Laboratory or Rideal-Walker method.

A careful study of the above tables will bring out the exceedingly important fact that, while slight differences in media used for growing the test organism previous to its employment in a test may cause serious discrepancies, the same differences in subculture media are of practically no significance. This has held true throughout all of our tests, so that it seems well demonstrated that, provided one has a uniform medium for growing the test culture, normal variations in the subculture medium are of little importance. It is true that differences in the subculture medium may cause a profound difference in the character of the chart obtained; that is, in the actual number of positive tubes shown in the subcultures from a given dilution. This influence, however, appears to be fairly uniform both for coal tar disinfectants and for the phenol control, so that there is no material change in the resulting coefficient.

In an effort to ascertain the cause of these variations in culture media the first consideration was, of course, the uniformity of the materials used in preparing the media. The salt used was in all cases the highest grade of C. P. product obtainable, so that it seemed safe to eliminate this factor from the problem. This leaves the water, beef extract, and Witte's peptone open to suspicion.

The Rideal-Walker method specifies the use of distilled water for the preparation of culture media, while the Hygienic Laboratory method specifies the use of tap water. Although it is claimed by some that tap water gives a medium more suited to the growth of bacteria, the writer, in view of the wide differences in the composition of various city water supplies as well as the seasonal and other variations occurring in a single supply, is convinced that its use is not desirable. A series of experiments performed by him extending over a period of several years indicated that media prepared with tap water were not in all cases comparable with those prepared with distilled water;

furthermore, the seasonal variations in the composition of the New York City water supply seemed to be sufficiently great to affect the uniformity of our phenol coefficient determinations. The only possible advantage in the use of tap water lies in its inorganic salt content. That a certain inorganic salt content is essential to any culture medium cannot be denied. This may, however, well be supplied by the use of sodium chloride in conjunction with the inorganic salts of the peptone and meat extract. Thus there seems to be no good reason for complicating the situation by employing tap water of unknown and variable composition. Throughout the work here reported distilled water was used in the preparation of all culture media. This factor could not, therefore, be concerned in any possible variation in the results obtained.

A careful study of the records described above has failed to show any indication that lack of uniformity in Witte's peptone was in any way responsible for the unreliable results often obtained with different lots of media. These results, which now include upwards of twenty-five different lots of peptone, together with the experiments to be described later, seem to show with reasonable certainty that for purposes of standardization Witte's peptone is a reliable and uniform product.

Liebig's Extract of Meat, on the other hand, does not appear to be nearly so satisfactory. Several jars have been found which invariably produced a culture medium giving very high coefficients. The proportions of salts and other ingredients of meat extract undoubtedly vary from time to time and are possibly responsible for some of the variations in culture media. In the writer's opinion, however, the greater part of the difficulty is caused by the presence in some jars of a considerable amount of fat. Chemical examinations have shown that those jars giving high coefficients contained over 50 per cent more ether extractive than the average for Liebig's extract of meat. The usual process of boiling culture media with caustic soda would convert this fat, partially at least, into soap. The powerful antiseptic action of soap, even in very high dilutions, is well known and is probably sufficient to explain the high results

obtained with these jars of extract. If, as will be recommended in this paper, the media be left with acidity unadjusted the presence of fat will have no effect; furthermore, it appears to be only an occasional lot of extract which proves unsatisfactory, so that if each new lot be checked against a disinfectant of known strength this source of error may be eliminated.

When we come to the actual manipulations used in the preparation of culture media, an extremely complex and involved problem is presented. This question is largely one of the influence of the kind and amount of acidity upon the growth of the test organism as well as upon the composition of the medium itself during the various stages of its preparation. Clark, in a recent article in the Journal of Infectious Diseases, July, 1915, clearly shows the fallacy of our present titrimetric methods. He points out the important influence of the hydrogen ion concentration upon the viability of bacteria, as well as upon the chemical composition of media, and shows conclusively that our present methods of adjusting the acidity of culture media by phenolphthalein give absolutely no information upon this all-important point.

The Rideal-Walker and Hygienic Laboratory broths form excellent examples of the differences in actual acidity (hydrogen ion concentration) which may be expected when media of different composition are brought to the same reaction with phenolphthalein by our present methods. The Rideal-Walker broth contains 20 grams of meat extract, 20 grams of peptone, and 10 grams of salt; while the Hygienic Laboratory broth contains 3 grams of meat extract, 10 grams of peptone and 5 grams of salt. The titration curves of these two solutions are shown below in Chart I.

The curves show the progressive changes in the hydrogen ion concentration of these solutions caused by successive additions of acid or alkali. Assuming the value $P_H = 8.5$ as the neutral point of phenolphthalein and calculating both from the point at which the curves cross this line we find that when the reaction has been brought to $+1.5$ by the usual method, the Rideal-Walker broth would have a hydrogen ion concentration of ap-

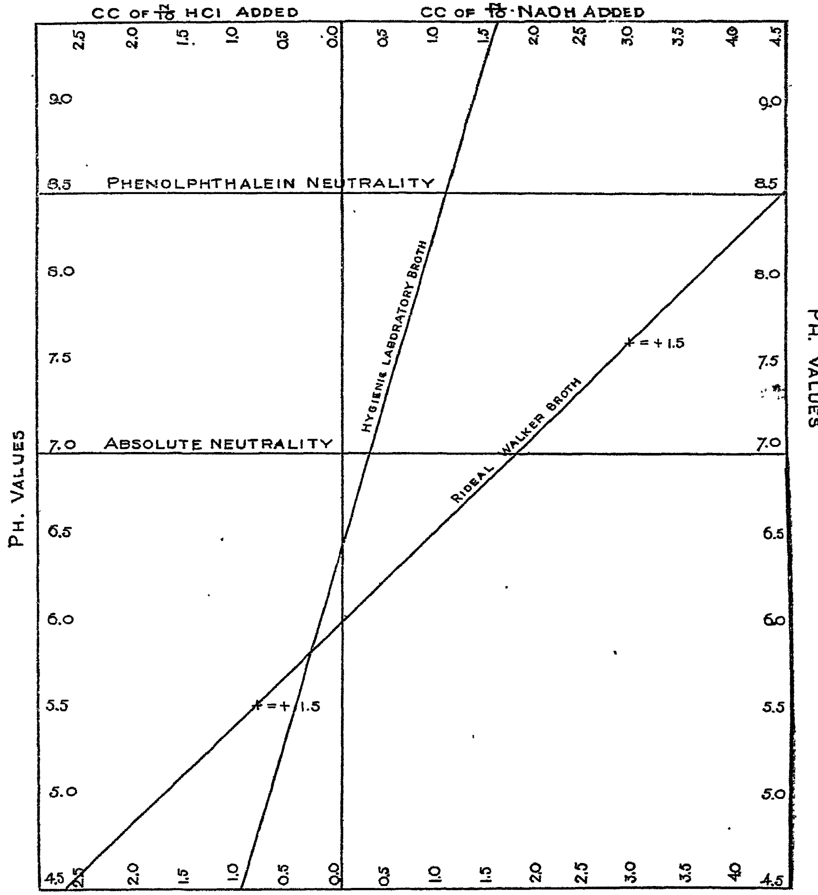


CHART 1. TITRATION CURVES OF 10 CC. OF HYGIENIC LABORATORY AND 10 CC. OF RIDEAL-WALKER BROTHS

The hydrogen ion concentrations given in this paper are expressed in the P_H values of Sörensen, (Compt. rend. du Lab. Carlsberg, 1909, **8**, p. 1.) The hydrogen electrode and a Leeds-Northrup potentiometer were used in the majority of the determinations. In some cases, however, the values given were obtained colorimetrically by means of the indicators, and essentially the same technique as described by Clark and Lubs (this Journal, vol. **2**, nos. 1, 2, and 3). We have made some rather extensive comparisons between this colorimetric procedure and the electrometric method as applied to the various media described in this paper and including the entire series of indicators recommended by Clark and Lubs. With all the sulphonaphthalein indicators the agreement has always been within 0.1 P_H. With methyl red, however, the results have been uniformly about 0.2 P_H more acid than those obtained with the hydrogen electrode. We believe, however, that for the majority of bacteriological purposes such an error is without significance and that the colorimetric method, carefully applied, constitutes a reliable and exceedingly convenient means for controlling the acidity of most types of culture media.

proximately $P_H = 7.65$ while that of the Hygienic Laboratory broth would be about 5.55. Due to the fact that few people judge the end point of phenolphthalein at exactly $P_H = 8.5$ the hydrogen ion concentration of media as ordinarily prepared varies somewhat from the above figures. This is particularly true of the Hygienic Laboratory broth in which the P_H value is usually very close to 5.0. Two media with such a wide difference in hydrogen ion concentration surely could not be expected to give comparable coefficients.

While the matter will be taken up in detail later, it may be well to mention here that the high results of the Rideal-Walker test as compared with the results of the Hygienic Laboratory test are not due to the difference in hydrogen ion concentration. The Rideal-Walker broth, with its 10 grams of NaCl in addition to the large amount introduced with 20 grams of meat extract, contains sufficient inorganic material to inhibit the growth of a weakened organism. If the salt be left out of the Rideal-Walker broth the results will be considerably lower than those obtained with the Hygienic Laboratory broth, in spite of the fact that the hydrogen ion concentration and titratable acidity of the Rideal-Walker broth are not changed.

While the above is sufficient to show the fallacy of attempting, by phenolphthalein titration, to adjust the reaction of culture media of different composition to the same point; there are many other points in our present methods of preparing culture media which make it practically impossible always to prepare even the same medium with a uniform hydrogen ion concentration. In a culture medium titrated with phenolphthalein there is no sharp end point. No two people will judge the "faint but distinct pink color" in exactly the same way. It is true that one person can, with practice, so train his eye as to obtain generally consistent results. There is no question, however, but that different workers, no matter how skillful and painstaking with their titration, cannot produce media of uniform reaction. This point is well illustrated in table 3.

After solution of the ingredients, a batch of Hygienic Laboratory medium was divided into three equal portions and

TABLE 3

Variations in coefficients obtained in different observers

ADJUSTED BY	FINAL TITRATION BY			HYGIENIC LABORATORY PHENOL COEFFICIENT OF DISINFECTANT A
	A	B	C	
A.........	1.48	1 91	1.26	$\dfrac{\frac{1100}{80}+\frac{1600}{100}}{2}$ = 14.75 average of 4 tests
B.........	1.21	1.56	1.08	$\dfrac{\frac{1100}{80}+\frac{1500}{100}}{2}$ = 14.37 average of 4 tests
C.........	1.75	2.17	1.41	$\dfrac{\frac{1300}{80}+\frac{1700}{100}}{2}$ = 16.62 average of 4 tests

each of three bacteriologists asked to adjust the acidity of one portion. After the media were completed each man was asked to determine the final reaction of the three lots without in any case knowing the identity of any of the samples. These results are shown above, together with the Hygienic Laboratory coefficients of coal tar disinfectant A resulting from the use of these media. The hydrogen ion concentration of the media was as follows.

Medium adjusted by bacteriologist A = 5.9
Medium adjusted by bacteriologist B = 5 6
Medium adjusted by bacteriologist C = 4.7

In the above determinations, as well as in all such tests reported in this paper, the medium used for growing the test culture and for subcultures was the same. The test organism was transferred daily for seven days and no longer, upon the particular medium indicated before being employed for a test. Great care was taken to have the test cultures in all cases exactly twenty-four hours old. We consider this an important point and are convinced that a few hours difference in the age of the test cultures has a very marked influence upon the uniformity of results. In order to eliminate every possible chance of variation, one jar of Liebig's extract of meat and one bottle

of Witte's peptone were set aside for use exclusively with the experimental media, so that all media described in this paper were made from the same jar of extract and the same bottle of peptone, unless otherwise indicated. In no case have conclusions been based upon the results of less than four tests, and usually six or more duplicate tests were made on different days and with different lots of media.

TABLE 4

Variation in coefficients with different methods of preparing media

Hygienic Laboratory technique

DISINFECTANT	DILUTION	MEDIUM: STANDARD R.-W. BROTH						MEDIUM: R.-W. BROTH, REDUCED TO + 1.5 WITHOUT NUETRALIZING					
		Minutes of exposure											
		2½	5	7½	10	12½	15	2½	5	7½	10	12½	15
A	1: 900							–	–	–	–	–	–
	1: 1000							+	–	+	–	–	–
	1: 1200							+	+	+	–	–	–
	1: 1300							+	+	+	+	+	+
	1: 1400	–	–	–	–	–	–	+	+	+	+	+	+
	1: 1600	+	–	–	–	–	–	+	+	+	+	+	+
	1: 1800	+	+	–	–	–	–						
	1: 2000	+	+	–	–	–	–						
	1: 2200	+	+	+	–	–	–						
	1: 2400	+	+	+	+	+	+						
Phenol	1: 80	–	–	–	–	–	–	–	–	–	–	–	–
	1: 90	+	–	–	–	–	–	+	+	–	–	–	–
	1: 100	+	+	–	–	–	–	+	+	+	+	–	–
	1: 110	+	+	–	+	–	–	+	+	+	+	+	+
	1: 120	+	+	+	+	–	–	+	+	+	+	+	+
	1: 130	+	+	+	+	+	+						
Coefficient		17.91						11.62					
P_H		7.8						8.4					

The Hygienic Laboratory method states simply that the medium should be adjusted to a reaction of + 1.5. A simple solution of the ingredients for the Hygienic Laboratory broth has an initial reaction of about + 0.9. Should this solution be neutralized and then brought up to + 1.5, or should sufficient acid be added to bring the reaction to the desired point without

neutralization? The Rideal-Walker broth has an initial acidity of + 3.5. This method specifies that the medium shall be neutralized and then 15 cc. of HCl per liter shall be added. It would seem therefore that there could be no excuse for simply adding sufficient alkali to bring the reaction down to the desired point.

TABLE 5

Variation in coefficients with different methods of preparing media
Hygienic Laboratory technique

DISINFECTANT	DILUTION	MEDIA: H.-L. BROTH, NEUTRALIZED AND THEN RAISED TO + 1.5 WITH HCl						MEDIA: H.-L. BROTH, RAISED TO + 1.5 WITH HCl WITHOUT NEUTRALIZATION					
		Minutes of exposure											
		2½	5	7½	10	12½	15	2½	5	7½	10	12½	15
	1:1000	−	−	−	−	−	−	−	−	−	−	−	−
	1:1100	−	−	−	−	−	−	+	−	−	−	−	−
	1:1200	−	−	−	−	−	−	+	+	−	−	−	−
	1:1300	+	−	−	−	−	−	+	+	+	−	−	−
A	1:1400	+	+	+	−	−	−	+	+	+	+	−	−
	1:1500	+	+	+	+	−	−	+	+	+	+	+	−
	1:1600	+	+	+	+	−	−	+	+	+	+	+	+
	1:1700	+	+	+	+	+	−	+	+	+	+	+	+
	1:1800	+	+	+	+	+	+	+	+	+	+	+	+
	1: 80	−	−	−	−	−	−	−	−	−	−	−	−
	1: 90	−	−	−	−	−	−	+	−	−	−	−	−
Phenol	1: 100	+	+	−	−	−	−	+	+	+	−	−	−
	1: 110	+	+	+	+	−	−	+	+	+	+	+	+
	1: 120	+	+	+	+	+	+	+	+	+	+	+	+
Phenol coefficient		14.94						13.75					
P_H value		5.2						5.7					

The effects of such differences in the preparation of culture media are illustrated by tables 4 and 5. In obtaining the results shown in these tables a single batch of Hygienic Laboratory broth and one of Rideal-Walker broth were prepared. Before adjusting the acidity, each lot was divided into two portions, one portion being neutralized with NaOH and then without filtration brought up to a reaction of + 1.5 with HCl, while to the other portion sufficient acid or alkali, as the case might be, was added to bring the reaction to the desired point. The differ-

ences in hydrogen ion concentration caused by such a variation in the method of preparing the media are also shown in the table. While this difference is not great, it is undoubtedly the cause of at least part of the discrepancies shown in the coefficients. It is, however, probable that a greater effect is produced by the changes in composition caused by the different methods of preparation.

When any medium is neutralized with alkali a considerable precipitate forms which only partially redissolves upon the addition of acid. This precipitate which separates out on the addition of acid or alkali consists of protein material and a considerable amount of phosphate, and its removal has a decidedly deleterious influence upon the subsequent growth of organisms. The hydrogen ion concentration of the medium is materially raised, due probably to the removal of "buffers." The increased hydrogen ion concentration, together with the reduced nutritive value, and the absence of phosphate, result in a very poor growth of the typhoid organism. On the finished culture being used for tests the results are not only much too high but the charts obtained are very irregular and unsatisfactory. This point is well illustrated by table 6. As would be expected, this phenomenon is more marked with the Hygienic Laboratory broth than with the Rideal-Walker medium.

It has probably been the experience of every worker that the amount of alkali indicated by titration is never sufficient to bring about complete neutralization of the media, it being necessary to add a considerable excess over the amount indicated. There will be a very decided difference in the coefficient obtained, depending upon how close to the neutral point the reaction has been carried before the addition of acid.

As previously mentioned, the Rideal-Walker method specifies that, after the medium has been neutralized, 15 cc. of N/1 HCl per liter shall be added. This should give the final product a reaction of + 1.5, but the acidity is always considerably less than the standard. Wide variations in hydrogen ion concentration with resulting variations in coefficients are obtained, depending upon whether or not an attempt is made to correct the medium to exactly 1.5.

In order to ascertain, if possible, just what influence variations in hydrogen ion concentration have upon culture media, a series of experiments was performed with media of varying P_H values. In these tests the proportion of the meat extract and peptone was as specified in the Hygienic Laboratory method, and in most cases the P_H values were adjusted by simply adding sufficient

TABLE 6

Variation in coefficients with different methods of preparing media

Hygienic Laboratory technique

DISINFECTANT	DILUTION	MEDIUM: H.-L. BROTH, NEUTRALIZED AND BROUGHT UP TO + 1.5						MEDIUM: H.-L. BROTH, NEUTRALIZED, FILTERED AND THEN BROUGHT UP TO + 1.5					
		Minutes of exposure											
		$2\frac{1}{2}$	5	$7\frac{1}{2}$	10	$12\frac{1}{2}$	15	$2\frac{1}{2}$	5	$7\frac{1}{2}$	10	$12\frac{1}{2}$	15
A	1:1200	–	–	–	–	–	–						
	1:1400	+	+	–	–	–	–						
	1:1600	+	+	+	+	–	–	–	–	–	–	–	–
	1:1800	+	+	+	+	+	+	+	–	–	–	+	–
	1:2000	+	+	+	+	+	+	+	+	–	–	–	–
	1:2200							+	+	–	–	+	–
	1:2400							–	+	+	+	–	–
	1:2600							+	+	+	+	+	+
Phenol	1: 80	–	–	–	–	–	–						
	1: 90	+	–	–	–	–	–						
	1: 100	+	+	+	–	–	–	–	–	–	–	–	–
	1: 110	+	+	+	+	+	–	+	–	–	–	–	–
	1: 120	+	+	+	+	+	+	+	+	–	+	–	–
	1: 130							+	+	–	–	+	–
	1: 140							+	+	+	+	+	+
Coefficient		14.77						17.72					
P_H		5.26						4.92					

NaOH or HCl, to bring the reaction to the desired point. Considerable work, however, was done in which the P_H value was adjusted by the use of phosphoric, lactic and citric acids, and sodium acid phosphate. Little difference could be detected in media prepared with different acids, provided the hydrogen ion concentration was uniform. In fact, all our work pointed to the conclusion that ordinary variations in the character and amount

of titratable acid present are of little importance, except in so far as they influence the P_H value.

The method of preparing the medium was as follows. The peptone, meat extract, and salt were boiled 15 minutes in the proper amount of distilled water. Then sufficient acid or alkali, as the case might be, was added to bring the P_H value to the desired point, the medium then being sterilized under 10 pounds pressure for twenty minutes. Different lots of medium were prepared in which the P_H values increased regularly by one-half unit from 8.5 to 4.5, the entire series being repeated three times. The relative amount of precipitate in each lot of medium was observed. A precipitate always formed upon the addition of more than a trace of alkali, and usually upon the addition of acid. In some instances the precipitate was not observable at once, but was present after the medium had been sterilized and allowed to cool. In all cases the amount of precipitate recorded is the amount present in the finished product.

It was found that the solubility of the peptone and meat extract was largely dependent upon the hydrogen ion concentration. This is graphically shown in curve 2, Chart II. When the P_H value fell between 6 and 7 no precipitate of any kind was present in the finished medium. A slight increase or decrease in acidity on either side of this point was however sufficient to cause a marked precipitation of the media. While the acidity of ordinary culture media probably never runs beyond $P_H = 4.5$, it is worthy of mention here that, when the P_H value is over 4, the peptone and meat extract again becomes perfectly soluble, the maximum precipitation occurring at approximately $P_H = 4.75$. It is also worthy of note that a chemical examination of these precipitates showed that that formed on the acid side of the chart consists almost entirely of protein, while that found on the alkaline side contains relatively large quantities of phosphates.[1]

[1] The writer feels that he should acknowledge his indebtedness to Dr. I. J. Kligler of the American Museum of Natural History, who, while discussing the results of some similar investigations of his own with an American peptone, first called the writer's attention to the fact that the solubility of peptone depended largely upon the hydrogen ion concentration of the solvent.

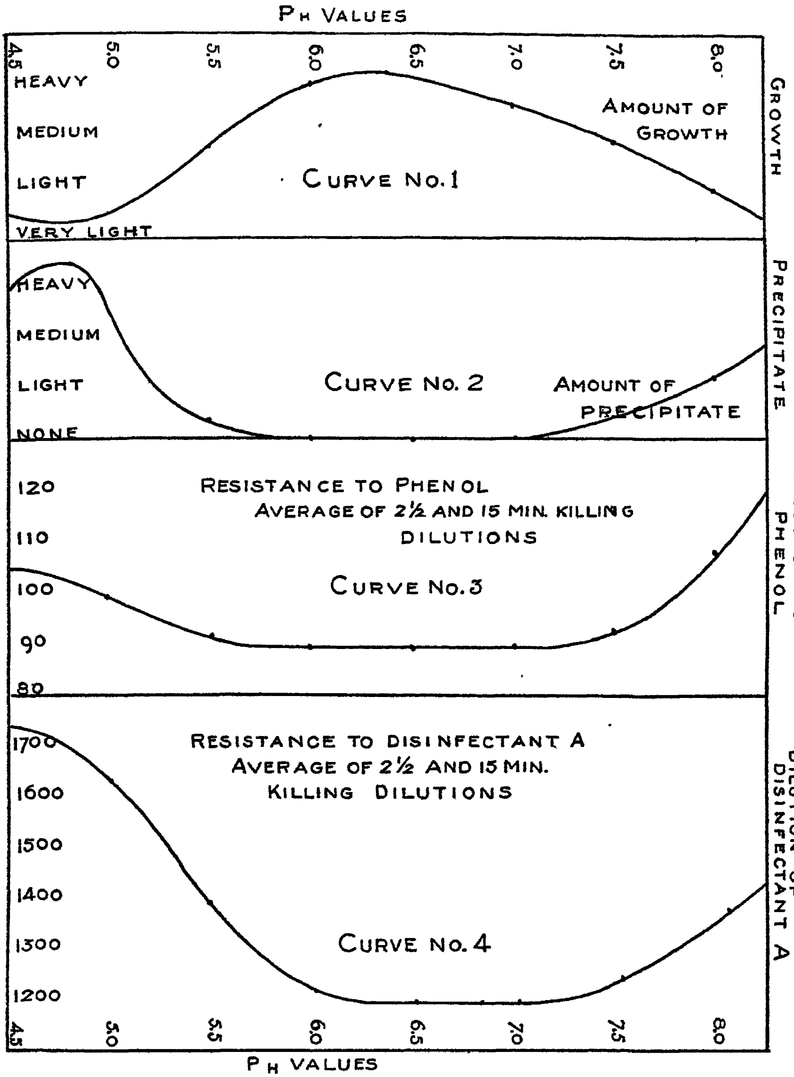


CHART 2

Examinations of a large number of different batches of media showed that the P_H value of standard Hygienic Laboratory broth varies from 4.75 to 5.75, while that of standard Rideal-Walker broth varies from 7.5 to 8.5. It will be seen therefore that the

composition of these media which is dependent largely upon a variable hydrogen ion concentration must consequently itself be variable. Such variations in chemical composition are undoubtedly one cause of the differences in the coefficients obtained with different lots of the same culture medium, although the indefinite hydrogen ion concentration is, of itself, without any reference to its influence upon solubility, an important point.

The development of the test organism in these media also forms an important index of their suitability for purposes of standardization. Five tubes of each broth were inoculated with *Bact. typhosum* Hopkins and were transferred daily for ten generations. These cultures were incubated at 37°C. and after twenty-four hours the amount of growth in each was recorded. The relationship between the amount of growth and the hydrogen ion concentration of the media is shown in curve 1, Chart II. The close agreement of this curve with that showing the relative amount of precipitate is quite remarkable and, as might be expected, the most luxuriant growth occurs in the same zone as that in which the peptone and meat extract are completely soluble.

Finally, these media were employed in determinations of the phenol coefficient of a coal tar disinfectant. At least five tests were run with each kind of medium on different days, using the same medium for both subcultures and for preliminary cultivation of the test organisms. The resistance of *Bact. typhosum* Hopkins in its relation to the various P_H values of the media is shown in curves 3 and 4, Chart II.

The curves plotted in each case were obtained by taking the mean of the two and one-half and 15 minute killing dilutions, as shown by the average result of not less than five check tests. It will be seen that the general outline of these curves corresponds with the two previous ones, the most important point being that between $P_H = 6$ and $P_H = 7$ the resistance of the organism is strong and constant, as indicated by the flatness of the curve. As the acidity is increased above $P_H = 6$ the resistance to phenol decreases slightly, while the resistance to the coal tar disinfectant decreases very rapidly, resulting in a

large increase in coefficient. On the other hand, when the acidity is reduced below $P_H = 7$ the resistance to phenol increases more rapidly than the resistance to a coal tar disinfectant, the result being a lowered coefficient. We have no explanation to offer for this phenomenon unless it be the fact that with increased acidity a large amount of protein material, but no phosphate, is removed from the media, while with a decreased acidity a large amount of essential phosphate and a comparatively small amount of protein is removed.

From these results it would seem, however, that we are at least justified in assuming that in order to obtain uniform results the hydrogen ion concentration of a culture medium should be between $P_H = 6$ and $P_H = 7$. This is further borne out by a study of the uniformity of coefficients obtained in duplicate tests with media of the same hydrogen ion concentration. Our results show that between $P_H = 6$ and $P_H = 7$ our phenol coefficients varied less than 10 per cent; between $P_H = 4.5$ and $P_H = 5.5$ our phenol coefficients varied from 30 to 40 per cent; while between $P_H = 7.5$ and $P_H = 8.5$ our coefficients varied from 20 to 25 per cent. In this connection it is of importance to note that a simple solution of the ingredients with no adjustment of the acidity whatever gives a P_H value most favorable for uniform results, namely, 6.3 to 6.7. It would seem, therefore, that there is no legitimate excuse for spoiling a good thing by juggling the reaction.

The Hygienic Laboratory broth has been often criticised on the ground that it contains insufficient nutriment for the normal growth of the typhoid organism, resulting in weakened and unreliable cultures. That this is not true is well demonstrated by our results, for when the hydrogen ion concentration of the culture medium is approximately $P_H = 6.5$ and the formula is as specified in the Hygienic Laboratory method *Bact. typhosum* grows luxuriantly and the cultures have been found to possess a remarkable degree of uniformity in their resistance to the action of disinfectants. The light, variable cultures of *Bact. typhosum* obtained with the regular Hygienic Laboratory broth are due not to a lack of a sufficient amount of nutriment, but to

an altered composition and high acidity brought about by a misguided attempt to adjust the reaction. However, it was thought wise to determine the effect of varying the proportion of different ingredients in culture media. A series of experiments was therefore performed as indicated in the following tables. In this work no attempt was made to adjust the acidity, the ingredients being simply dissolved, filtered, and sterilized; otherwise the details throughout the procedure were exactly the same as those previously employed.

TABLE 7

Showing effect upon culture media of varying amounts of Witte's peptone. All media in this table contained Liebig's Meat Extract, 3 grams, and NaCl, *5 grams, to each 1000 cc. of distilled water, in addition to the amount of peptone indicated*

AMOUNT OF WITTE'S PEPTONE PER LITER	PH VALUE	TITRATABLE ACIDITY TO PHENOLPHTHALEIN	RELATIVE AMOUNT OF GROWTH SHOWN IN PRELIMINARY CULTURES	RESISTANCE TO DISINFECTANT AVERAGE OF $2\frac{1}{2}$ AND 15 MINUTE KILLING DILUTIONS		HYGIENIC LABORATORY COEFFICIENT OF DISINFECTANT A*
				Phenol	Disinfectant A	
grams.						
5	6.6	+0.75	Medium	1:95	1:1500	15.77
10	6.5	+0.93	Heavy	1:90	1:1200	13.33
15	6.4	+1.12	Heavy	1:89	1:1200	13.48
20	6.4	+1.35	Heavy	1:88	1:1190	13.52
25	6.3	+1.51	Heavy	1:89	1:1190	13.37
30	6.3	+1.74	Heavy	1:91	1:1210	13.29

* Average results of five check tests.

Table 7 shows the influence of adding varying amounts of Witte's peptone to culture media, the proportions of the other ingredients remaining constant. One medium was prepared containing no peptone. In this medium *Bact. typhosum* grew very poorly indeed, so that it was not considered worth while to continue the experiment. With 5 grams of the peptone a good growth of the test organism was obtained. The resistance of the organism was, however, markedly weaker than when grown in any other medium in this series. The use of amounts of Witte's peptone varying from 10 to 20 grams per liter made no apparent difference in the growth of the organism nor in

its relative resistance to the action of disinfectants. It would seem, therefore, that there is no good reason for using a larger quantity of this ingredient than is now called for by the Hygienic Laboratory method.

That some meat extract is essential is shown by the results obtained with the first medium in table 8. Where no extract was used it was impossible to obtain more than an exceedingly light growth of the test organism, and when such a culture was used for testing a disinfectant the coefficient was not only

TABLE 8

Showing effect upon culture media of varying amounts of Liebig's extract of meat. All media in this table contained Witte's peptone, 10 grams and NaCl, *5 grams, to each 1000 cc. of distilled water in addition to the amount of meat extract indicated*

AMOUNT OF LIEBIG'S EXTRACT OF MEAT PER LITER	P_H VALUE	TITRATABLE ACIDITY TO PHENOLPHTHALEIN	RELATIVE AMOUNT OF GROWTH SHOWN IN PRELIMINARY CULTURES	RESISTANCE TO DISINFECTANT AVERAGE OF 2½ AND 15 MINUTE KILLING DILUTIONS		HYGIENIC LABORATORY COEFFICIENT OF DISINFECTANT A*
				Phenol	Disinfectant A	
grams						
None	6.9	+0.29	Very light	1:99	1:1710	17.27†
3	6.5	+0.81	Heavy	1:89	1:1190	13.37
5	6.4	+1.14	Heavy	1:91	1:1280	14.06
10	6.2	+1.81	Heavy	1:93	1:1380	14.85
15	6.2	+2.62	Heavy	1:93	1:1490	16.02
20	6.1	+3.44	Medium	1:95	1:1510	16.52
25	6.1	+4.23	Medium	1:97	1:1650	17.01

* Average results of five check tests.
† Very irregular.

very high but the charts obtained were very irregular and unsatisfactory in every particular. With 3 grams of meat extract the growth of the test organism was fully as good as that obtained with any medium tested. Its resistance to the action of disinfectants was strong and uniform, so that there was no difficulty in getting satisfactory checks with duplicate tests. As the amount of meat extract was increased there was, apparently, a steady decrease in the resistance of the test culture, and as this was more pronounced in the case of the coal tar disinfect-

ants than with phenol there was a corresponding increase in the phenol coefficient. This weakened resistance, however, is more apparent than real and is due largely to the fact that media containing large percentages of meat extract are sufficiently antiseptic to inhibit the growth of an organism which has been weakened but not killed by exposure to disinfectants. This is shown by a series of experiments in which the second medium in this table was used for subcultures with test cultures grown in each of the media shown in the table. In these experiments there was also an increase in coefficient with an increased percentage of meat extract. This, however, was only about one-

TABLE 9

Showing effect upon culture media of varying amounts of NaCl. *All media in this table contained Witte's peptone, 10 grams and Liebig's Extract of Meat, 3 grams, to each 1000 cc. of distilled water in addition to the amount of* NaCl *indicated*

AMOUNT OF NaCl PER LITER	PH VALUE	TITRATABLE ACIDITY TO PHENOL-PHTHALEIN	RELATIVE AMOUNT OF GROWTH SHOWN IN PRELIMINARY CULTURES	RESISTANCE TO DISINFECTANT AVERAGE OF 2½ AND 15 MINUTE KILLING DILUTIONS		HYGIENIC LABORATORY COEFFICIENT OF DISINFECTANT A*
				Phenol	Disinfectant A	
grams						
None	6.6	0.93	Medium	1:94	1:1410	15.00
5	6.5	0.93	Heavy	1:90	1:1191	13.22
10	6.5	0.94	Medium	1:94	1:1480	15.74
15	6.4	0.93	Medium	1:96	1:1590	16.56
20	6.3	0.94	Light	1:98	1:1670	17.02

* Average result of five check tests.

half that obtained when the higher amounts of meat extract were introduced in the subculture media as well as in that used for preliminary cultivation. Moreover, this inhibitive action seems to vary materially with different lots of Liebig's extract of meat, so that it would seem wise to reduce this variable factor to a minimum by employing for a standard method as small an amount of meat extract as is consistent with the needs of the organism. From our results it seems clear that 3 grams per liter is ample.

It will be noted that in table 8 there is a very marked increase in titratable acidity when the larger amounts of meat extract

are used and at the same time there is a slight increase of the hydrogen ion concentration. It may possibly be contended that the inhibitive influence of the meat extract is due to this increased acidity. Such, however, is not the case as was shown by the fact that when the titratable acidity of these media was in all cases reduced to + 1.0 the resulting coefficients, while somewhat lower as would be expected with the decreased P_H value, still showed the same steady increase with increased percentages of meat extract.

That some salt is desirable in culture media is shown by the results in table 9, the best growths and the most resistant cultures being obtained with the medium containing 0.5 per cent. As the percentage of salt was increased the growth became less luxuriant with a corresponding increase in the phenol coefficient. As in the case of meat extract, the increased coefficient is partly due to the inhibitive action of the larger amount of salt upon the exposed organism rather than to an actual lowering of its resistance. In this connection it is of interest to note that, as Liebig's Meat Extract contains over 30 per cent of inorganic salts and Witte's peptone 3 to 10 per cent, a medium made up of 3 grams of the former, 10 grams of the latter, and 5 grams of NaCl would have an inorganic salt concentration closely approximating that of physiological salt solution. This is significant in view of the fact that this medium, of all those tested, has been the most favorable for the growth of the *Bact. typhosum* and has in every respect given the most uniform and satisfactory results.

A medium such as the above, in which no attempt is made to adjust the acidity, always has, in our experience, a P_H value of 6.3 to 6.6 which has been shown to be within the zone of hydrogen ion concentration most conducive to uniform and reliable results. The percentages of peptone, meat extract, and salt are those which have been found most favorable for a normal unrestricted growth of the test organism, as well as least liable to introduce disturbing variations. The phenol coefficient obtained with this medium is considerably lower than that given by either of the old methods, and hence may be considered un-

desirable by some manufacturers of disinfectants. However, if all manufacturers would guarantee their products upon the same basis, it is hard to see where anyone would suffer an injustice.

The most important need in a standard method for control of disinfectants is, of course, a reliable procedure for the accurate determination of the relative germicidal strength of disinfectants of the same class, and it makes no particular difference whether the results be high or low, so long as the results are uniform and the actual relative differences between different products are clearly shown. This seems to be easily accomplished if a culture medium such as the above be employed. Up to the present time more than 150 different determinations of phenol coefficients upon several disinfectants have been made with this medium, including the use of 50 different batches of media and 12 lots each of Witte's peptone and Liebig's extract of meat. The results of these tests have been very satisfactory throughout; the experimental error shown by duplicate tests has never exceeded 8 per cent and has almost always been much less than 5 per cent. It is hoped that, provided these results are confirmed, such a culture medium may be adopted as an official standard.

The writer is of the opinion that it is of little importance whether one employs the Hygienic Laboratory or the Rideal-Walker technique, provided a proper culture medium be used; personally, we are slightly inclined to favor the Hygienic Laboratory procedure and feel that the results obtained with it are slightly more uniform than with the Rideal-Walker. It is also quite possible that some entirely new technique may be developed which will prove superior to either of the present procedures. Whether old or new technique finally prevail, a reliable culture medium is the first essential.

Witte's peptone, owing to present European conditions, is very hard to obtain, and the small quantities available are almost prohibitive in price. As it is uncertain when a new supply will be available upon the American market, it would be of the utmost value if a product of American manufacture could

be found which might safely be substituted for Witte's. In order that data on this subject might be available for the use of the Committee on Standardization of Disinfectants of the American Public Health Association a study of various American peptones was undertaken, in conjunction with Prof. Earle B. Phelps, Chairman of the above committee.

The different manufacturers were asked to submit samples representing as many different batches of their product as possible. In all, fifteen samples from four manufacturers were received, all of which were examined in comparison with Witte's peptone. For this work media were prepared from the different peptones, using the formula previously described as having given the most uniform results with Witte's; namely, 10 grams of peptone, 3 grams of Liebig's extract, and 5 grams of salt to each liter of distilled water, no attempt being made to adjust the acidity. The details of the experiments were in each case the same as those employed in the previous work. The influence of the different peptones upon the resistance of *Bact. typhosum* to phenol and to a coal tar disinfectant is shown graphically in Chart III. The solid portions represent those dilutions which kill in two and one-half minutes, while the shaded portions represent those dilutions which kill at fifteen minutes. The results of four separate tests with each lot of peptone are shown. The hydrogen ion concentrations, as well as the titratable acidities to phenolphthalein, are indicated on the chart. For comparative purposes similar results obtained with different lots of Witte's peptone are also included.

It will be noted that the hydrogen ion concentrations of the medium prepared with Armour's peptone varied from $P_H = 5.9$ to $P_H = 6.3$, thus practically falling within the zone where our previous work would lead one to expect uniform and reliable results. With these media the preliminary growth of the test organism was luxuriant. The resistance of the test organism, both to the action of phenol and to disinfectant B is remarkably uniform and in close agreement with the results obtained with Witte's peptone. In so far as can be determined from the limited number of samples examined, different lots of Armour's

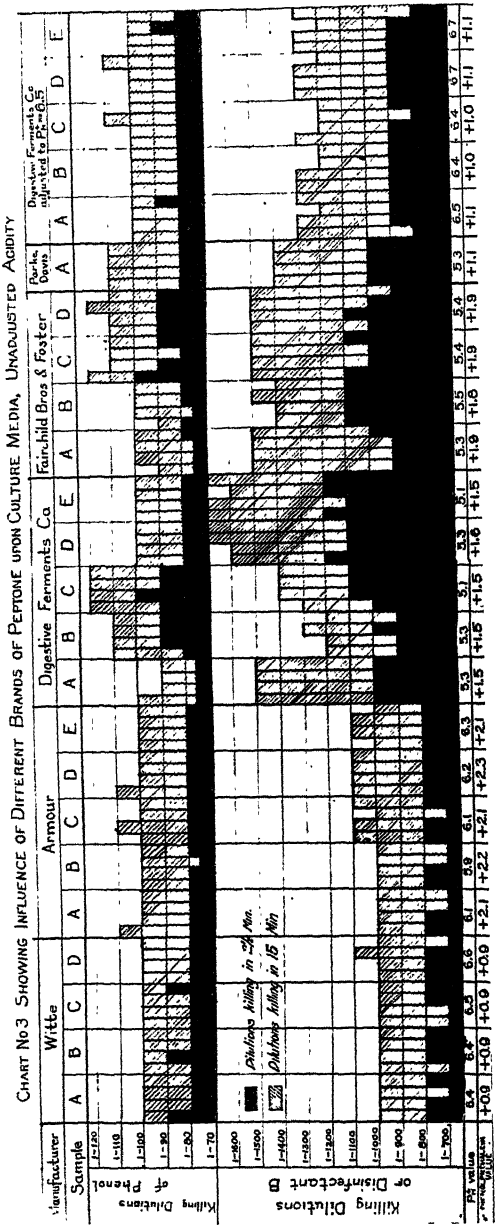


Chart No 3 Showing Influence of Different Brands of Peptone upon Culture Media, Unadjusted Acidity
Manufacturer
Sample
Witte
Armour
Digestive Ferments Co
Fairchild Bros & Foster
Parke, Davis
Digestive Ferments Co adjusted to PH=6.5
Killing Dilutions of Phenol
Killing Dilutions of Disinfectant B
Dilutions killing in 2½ Min.
Dilutions killing in 15 Min.
PH value

peptone seemed to possess a sufficient degree of uniformity, so that in this medium the product may possibly prove a satisfactory substitute for Witte's peptone. It must be remembered, however, that only five samples were examined, and it is quite possible that other lots may not prove so satisfactory.

Media prepared from the 5 samples of peptone furnished by the Digestive Ferments Company had a hydrogen ion concentration of from 5.1 to 5.3; media prepared from the 4 peptones furnished by Fairchild Brothers & Foster had a hydrogen ion concentration of from 5.3 to 5.5; while that from the one sample furnished by Parke, Davis & Company[2] had a hydrogen ion concentration of 5.3. It will be seen, therefore, that the acidities of these media lie outside of the zone in which uniform results were obtained with Witte's peptone, and as might be expected from the high acidity the results obtained with these products were much less satisfactory than those obtained with Armour's. The resistance of the test culture to the action of phenol was irregular and as a rule quite low. The resistance to the action of disinfectant B was also irregular and at the same time was very much less than when either Armour's or Witte's peptones were used. It is of interest to note that while all of these products were practically completely soluble in cold water, there was almost invariably a heavy precipitate in the finished sterilized medium, and that the growth of *Bact. typhosum* obtained was in all cases much less luxuriant than that given by either Armour's or Witte's peptones. This checks up very well with our previous results, which indicated that a medium having a hydrogen ion concentration in the neighborhood of 5 was variable in composition and in every way unsatisfactory for a normal development of the test organisms.

It was thought desirable to determine the effect of reducing the hydrogen ion concentration of these media to a point approximating that given by Witte's peptone. For this purpose the titration curves of batches of media prepared from each of the

[2] Only one sample of peptone was furnished by this company and it is understood that the preparation of this product is in an experimental stage and that it is not being marketed.

Digestive Ferments Company's peptones were determined. From these curves the amount of alkali necessary to bring the P_H value to 6.5 was calculated. Various lots of media were then prepared in which the ingredients were mixed and the amount of alkali indicated by the titration curve added, after which the mixture was boiled for fifteen minutes, filtered, tubed, and sterilized. As in the previous experiments, these media were then employed in the determination of the phenol coefficient of disinfectant B. These results are also shown in Chart III together with the final hydrogen ion concentrations, which will be seen to approximate closely the desired point.

The test organism grew luxuriantly, the growth obtained being fully equal to that given with the unadjusted medium prepared from Witte's or Armour's peptone. There was no evidence of any precipitate in the finished media, and as will be seen from the chart the resistance of the test organism was very uniform in all cases, so that the actual variations in coefficient are negligible. It will be noted, however, that the resistance to disinfectant B is still somewhat weaker than was shown with either Witte's or Armour's peptone and as a result the phenol coefficient is somewhat higher. Thus while the adjustment to a P_H value of approximately 6.5 of media prepared from the Digestive Ferments Company's peptone enables one to obtain uniform coefficients, these coefficients are not strictly comparable with those obtained with Witte's and Armour's. It seems quite clear, therefore, that there are differences in peptone other than acidity which are capable of causing variations in the resistance of *Bact. typhosum* to the action of disinfectants. While we have not taken the time to repeat these experiments on Fairchild Brothers & Foster's, or Parke, Davis & Company's peptones, we see no reason why a similar test should not give similar results.

From these results it becomes apparent that with the possible exception of one peptone, the American products cannot be safely substituted for Witte's peptone under the above conditions. It should be borne in mind, however, that these results were obtained upon culture media in which no attempt was

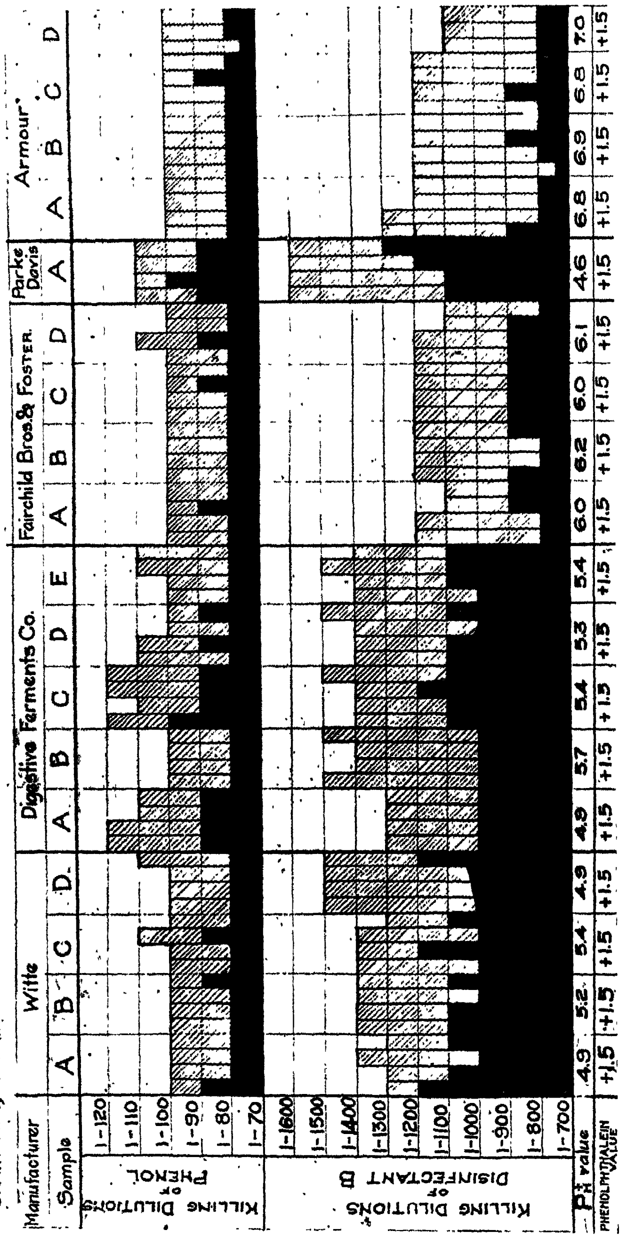
made to adjust the acidity. When the acidity is adjusted by first neutralizing and then bringing up to an acidity of + 1.5 to phenolphthalein entirely different results are to be expected. The results obtained with media adjusted in this way are shown in Chart IV.

A comparison of this chart with Chart III will bring out a remarkable difference in the behavior of the various peptones under these conditions. No better illustration could be desired of the futility of attempting to adjust the acidity of culture media to a definite point by phenolphthalein titration. Here we have sixteen different lots of culture media, all with a phenolphthalein acidity of + 1.5, in which the hydrogen ion concentration varies from $P_H = 4.6$ to $P_H = 7.0$. In the case of Witte's and Parke, Davis & Company's peptones, with which the initial titratable acidities were less than + 1.5, there is a marked increase in the hydrogen ion concentration. The media from Fairchild Brothers & Foster's and Armour's peptones, having an unadjusted acidity to phenolphthalein greater than + 1.5, show when adjusted to this point reduced P_H values. The Digestive Ferments Company's peptone, with which the initial phenolphthalein acidity was practically + 1.5, is not particularly affected except that there is a slightly greater variation in the P_H values of the adjusted media, which again illustrates the fallacy of our present method of adjusting the reactions of culture media.

Chart IV shows the same general relationship between the resistance of the organism to the action of disinfectants and the P_H value of the medium as was found with the unadjusted media given in Chart III. With the exception of greater variability in the phenol results, the resistance of the test organism when grown in media prepared with Digestive Ferments Company's peptone is practically identical with that shown by Witte's peptone. Fairchild Brothers & Foster's and Armour's peptones, owing to their low hydrogen ion concentration, cause a marked increase in the resistance of the test culture.

It will be noted that with adjusted culture media made from the Digestive Ferments Company's peptone the cultures are

CHART No. 4, SHOWING INFLUENCE OF DIFFERENT BRANDS OF PEPTONE UPON STANDARD HYGIENIC LABORATORY CULTURE MEDIA.



slightly more resistant and at the same time more uniform than with the unadjusted media. This certainly cannot be explained on the basis of hydrogen ion concentrations and the difference is too great to be laid to accident. This phase of the problem is worthy of careful study and involves painstaking investigations

TABLE 10

Showing phenol coefficients of disinfectant B obtained with different peptones

PEPTONE		AMOUNT OF GROWTH SHOWN IN PRELIMINARY CULTIVATION		PHENOL COEFFICIENT OF DISINFECTANT B AVERAGE RESULT OF FOUR TESTS	
		Medium adjusted	Medium unadjusted	Medium adjusted	Medium unadjusted
Witte	A	Light	Heavy	13.28	9.72
	B	Light	Heavy	13.45	9.72
	C	Light	Heavy	12.97	9.58
	D	Light	Heavy	13.28	10.00
Digestive Ferments Company	A	Medium	Light	11.21	14.92
	B	Light	Light	13.61	10.88
	C	Light	Light	12.75	11.52
	D	Light	Light	13.44	15.41
	E	Light	Light	13.78	15.83
Fairchild Brothers & Foster	A	Heavy	Medium	10.95	13.79
	B	Heavy	Medium	11.38	14.06
	C	Heavy	Medium	11.56	12.59
	D	Heavy	Medium	10.94	12.34
Armour	A	Heavy	Heavy	11.52	9.58
	B	Heavy	Heavy	11.04	9.79
	C	Heavy	Heavy	11.09	10.13
	D	Heavy	Heavy	10.58	10.41
	E	Heavy	Heavy		10.22
Parke, Davis & Company	A	Medium	Medium	13.21	12.70

into the chemistry of peptones. It does not, however, come within the scope of the present paper.

The average phenol coefficient obtained with each of the above peptones is shown above in table 10.

From a study of these results it seems clear that, with the possible exception of Armour's, none of the American peptones

could be safely substituted for Witte's in unadjusted culture media. The results obtained with Armour's peptone are fairly uniform and are in sufficiently close agreement with Witte's to warrant a more extended investigation of this product and to justify the hope that it may prove a satisfactory substitute for the imported peptone for use in unadjusted media, which we trust may soon be adopted as a standard for this work.

It will undoubtedly be some time, however, before such a change can be made in the official procedure. It would be of value, therefore, if an American product could be found which would give results comparable to and at least no worse than those given by Witte's peptone in the present Hygienic Laboratory media. A study of our results will show that the Digestive Ferments Company's peptone is the only one which could possibly fulfill these requirements and even with this the results are much less uniform than with Witte's. In justice to this product it should be stated, however, that the results given for Witte's peptone are from lots of media selected from a long series of tests and represent the average results which may be expected with this product, rather than the actual limits of variability. It should also be pointed out that these variations do not represent actual differences between the different samples of the same product. We have found in different batches of media made from the same bottle of Digestive Ferments Company's peptone variations fully as great as are shown here with different samples. The trouble does not appear to be due so much to actual variations in the peptone itself, as to the fact that with a high hydrogen ion concentration it is practically impossible to control the procedure so as always to produce solutions of uniform characteristics, even from identical materials.

SUMMARY

The essential points brought out by this work may be summed up as follows.

1. Variations in culture media are the cause of the majority of the discrepancies obtained in the bacteriological examination of disinfectants.

2. There is no indication that these variations in culture media are in any way due to lack of uniformity in Witte's peptone. Liebig's Extract of Meat, however, should be regarded with suspicion.

3. The greater part of the difficulty lies in our present methods of adjusting the acidity.

4. The hydrogen ion concentration of a culture medium has important influences upon its composition and upon its suitability for the growth of *Bact. typhosum*.

5. There is a marked relationship between the hydrogen ion concentration of the culture medium and the resistance of the test organism to the action of disinfectants.

6. The most satisfactory and uniform results have been obtained with a culture medium in which the P_H value falls between 6 and 7. This condition is easily obtained with a medium containing 10 grams of Witte's peptone, 3 grams of Liebig's meat extract, and 5 grams of salt, boiled fifteen minutes, filtered, tubed, and sterilized, with no attempt to adjust the acidity.

7. It has been found that 3 grams of meat extract are ample and that an increased amount is liable to cause disturbing variations.

8. It has also been found that 10 grams of Witte's peptone is sufficient for all the needs of the test culture.

9. The best results have been obtained with a medium containing 5 grams of salt per liter, an increase of salt content causing an increased coefficient due to the inhibitive action of the salt upon the exposed organism.

10. Of the different brands of American peptone only one has been found which could possibly be safely substituted for Witte's in the unadjusted medium. Insufficient samples, however, have been tested to enable one to make this statement with certainty.

11. The Digestive Ferments Company's peptone gives results most closely approximating those of Witte's when used in standard Hygienic Laboratory media.

In conclusion it should be stated that this work was made

possible by the financial support and interest of the West Disinfecting Company through their chemist, Dr. W. Dreyfus. The writer's appreciation is also due to Dr. H. D. Pease of the Lederle Laboratories for his interest in the work, and to Prof. E. B. Phelps of the Hygienic Laboratory, Washington, D. C., who very kindly had many determinations of hydrogen ion concentration made in his laboratory.

Since this article was written the Digestive Ferments Company and Fairchild Brothers & Foster have submitted new samples of peptone based upon the results of our experimental work. In these peptones they have attempted to standardize the hydrogen ion concentration so that the finished media with unadjusted acidity will have a desirable P_H value. We are at present examining these products, and while we have not as yet done a sufficient amount of work to warrant drawing definite conclusions, the present indications are that the new products will prove much more satisfactory than the old ones.

STUDIES ON THE NOMENCLATURE AND CLASSIFICATION OF THE BACTERIA

III. THE FAMILIES OF THE EUBACTERIALES

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The order Eubacteriales may be characterized as follows:

Order 1. **Eubacteriales,** ordo nov.

Synonyms:

Bacterina Perty (1852, p. 179, in part)
Eubacteria Schroeter (1882, p. 154)
Eubacteria Migula (1900, p. 1)
Eubacteriaceae A. J. Smith (1902, p. 270)
Haplobacteriacei Fischer (1895), in part
Haplobacterinae Fischer (1903), in part

This order includes the true bacteria, those forms which are least differentiated and least specialized. The cells are usually minute, spherical, rodshaped or spiral, not typically producing true filaments although the cells may occur in chains or other groups. The cells do not have a well-organized or differentiated nucleus. They may be motile by means of flagella, or non-motile, never notably flexuous. Multiplication by transverse fission, never by longitudinal. Some forms, particularly rod-shaped types, produce endospores, but never conidia. Branching of cells occurs in a few forms which intergrade with the Actinomycetales. Neither sulphur granules nor bacteriopurpurin are present, though the cells may be pigmented. Chlorophyll is absent (with the possible exception of one genus.) The cells may be united into gelatinous masses, but never form motile pseudoplasmodia, nor develop a highly specialized cyst-producing fruiting stage.

With very few exceptions, authors have recognized that the group here termed the order *Eubacteriales* should be divided into three subgroups or families on the basis of the shape of the cell, that is, into cocci, bacilli and spirilla. Cohn (1872) used the names *Sphaerobacteria, Microbacteria, Desmobacteria* and *Spirobacteria* to designate the cocci, the short rods, the long rods and the spirilla respectively. Zopf (1883) used the names *Coccaceen* and *Bacteriaceen,* later (1885) including the spiral forms with the rods under the second family. Flügge (1886) termed the three groups *Mikrobacterien, Bacillen* and *Spirillen.* Schroeter (1886) used *Coccacei* and *Bacteriacei* in the sense of Zopf. Trevisan (1885) and De Toni and Trevisan (1889) include all spherical organisms under *Coccogenae* and all rods and spirals under *Baculogenae.* Sternberg (1892) employed the terms *Micrococci, Bacilli* and *Spirilla.* Migula (1894, 1895, 1897, 1900, 1904) uses the family names *Coccaceae, Bacteriaceae* and *Spirillaceae.* In this nomenclature he is followed by Lehmann and Neumann (1896), Chester (1897, 1901), A. J. Smith (1902), Kendall (1902), E. F. Smith (1905), Schneider (1912), Engler (1912), and Vuillemin (1913).

Hueppe (1895) termed the families *Coccaceae, Bacteriaceae* and *Spirobacteriaceae.* Matzuschita (1902), Fischer (1903) and Flügge (1905), use *Coccaceae, Bacillaceae* and *Spirillaceae.*

The principal departure from this type of classification is that of Jensen. As previously noted, this author does not recognize a group corresponding to the *Eubacteriales.* The families which he lists that would fall under this group as here defined would be *Oxidobacteriaceae, Luminobacteriaceae, Reducibacteriaceae, Acidobacteriaceae, Alkalibacteriaceae, Butyribacteriaceae* and *Putribacteriaceae.*

It is evident from the names which have been used that most authors have adhered very strictly to morphology as a basis for separation of families, while Jensen has emphasized physiology. It would seem that wherever a morphological basis for grouping does not lead to the separation of closely related forms, and the inclusion of unlike types in the same group, it should be employed, but when such occurs another method of separation,

probably based upon physiological characters should be used. The unanimity with which the separation into families on the basis of shape has been used would indicate that in general it has proved fairly satisfactory. In a few cases it probably leads to somewhat anomalous results. For example, to any student of the lactic acid bacteria the separation of the lactic streptococci and bacilli into different families is difficult in view of the numerous intergradations. Perhaps even more striking is the inclusion in separate families of Winogradsky's *Nitrosococcus* and *Nitrosomonas* in most classifications. The organisms belonging to the two genera are evidently closely related, they are both prototrophic, oxidize ammonia to nitrates, and do not develop on ordinary media. It would seem that these genera together with *Nitrobacter* might well be grouped together as a distinct family.

Many of the names which have been proposed for these families do not conform to the rule of the botanical code which reads "Families are designated by the name of one of their genera or ancient generic names with the ending *aceae*." With this rule in mind and with due respect to priority, the family names may be designated as *Coccaceae*, *Bacteriaceae*, *Spirillaceae* and *Nitrobacteriaceae*.

Following is a key to these families, giving the principal characteristics.

Key to the families of the Eubacteriales

A. Organisms usually growing more or less readily upon organic media, not securing growth energy primarily by the oxidation of ammonia or nitrites.
- I. Cells typically spherical......................Family I. *Coccaceae*
- II. Cells not spherical, elongate.
  - a. Cells not spiral.....................Family II. *Bacteriaceae*
  - b. Cells spiral, or at least curved.......Family III. *Spirillaceae*

B. Not growing readily or at all on media containing considerable amounts of organic material; nitrifying bacteria, securing growth energy primarily by the oxidation of ammonia or nitrites.

Cells may be either spherical or rod-shaped,
Family IV. *Nitrobacteriaceae*

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THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRODUCTION OF PRECIPITATES IN A SOLUTION OF PEPTONE AND ITS RELATION TO THE NUTRITIVE VALUE OF MEDIA

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It is a well-known fact that when media are neutralized to phenolphthalein a precipitate is produced which may be partly redissolved by the addition of acid. If this precipitate is filtered off the nutritive quality of the medium is appreciably lowered. Likewise, experience has taught us that the optimum reaction of a culture medium is +1.0 to phenolphthalein or neutral to litmus. In neither case, however, has an adequate explanation been given for the observed facts.

From previous experiments on synthetic media it became evident that the significant nutritive factor in bacteriological culture media was the peptone and that a careful study of this substance should yield explanations of many puzzling facts. From various analyses it appears that petpone consists of proteoses, amino-acids and salts, the most important of the latter being the phosphates. The proteoses and complex nitrogenous compounds can not be utilized by the common bacteria but they do play an important part as buffers in the medium. The amino-acids are the essential nitrogenous foods upon which the nutritive value of the peptone depends. The phosphates furnish an important salt but are of even greater value as reaction regulators. An ideal peptone would, therefore, be one that contains these ingredients in the right proportions.

Since the reaction of the medium, both in the making and in the finished product, has so great an influence on the nutritive quality, attention was directed to the effect of different reactions,

in terms of hydrogen ion concentration, upon the peptone. Solutions of 1 per cent peptone in distilled water were made up and varying amounts of acid or alkali added to obtain a graded series of hydrogen ion concentrations. Solutions of Witte and Difco peptone were studied in this manner.

The results obtained bear so directly on the problem that even though the procedure was only qualitative they are of sufficient interest to deserve recording. Briefly stated, it was found that peptone has two precipitating zones, one on the acid side, the other on the alkali side, with an intermediate zone of complete solubility. In terms of P_H, peptone (Witte's) is completely soluble between P_H 6.8 and P_H 8.0. At P_H 8.2 precipitation begins and increases progressively to P_H 9.0. Similarly, a precipitation occurs at about 5.4 and increases up to about 5.0, then decreases again to about P_H 4.0 when no precipitate is formed.

The nature of the precipitate in the two zones is suggestive. At the alkali end the precipitate appears to consist largely of phosphates plus some organic constituents. The acid precipitate, on the other hand, is organic and when redissolved gives reactions characteristic of proteoses and peptone.

Both Witte's and Difco peptone behaved in the manner outlined with the following exceptions: Witte's peptone when dissolved in water has a P_H value of 6.5 to 6.8. It dissolves slowly and with a clear solution. On autoclaving only a slight sediment is sometimes obtained. The Difco peptone, on the other hand, has a P_H value of 5.1 to 5.4 when dissolved. It goes into solution readily but gives a heavy precipitate on autoclaving. This is to be expected since its reaction is right at the precipitating zone. Like Witte's peptone it has a clear solubility zone between P_H 7.0 and 8.0 and a precipitating zone beyond 8.0.

The bearing of these facts on the changes obtained in the making of media and on their nutritive quality is obvious. The rôle of peptone in culture media (either infusion or extract) is twofold. It furnishes nitrogenous food in the form of amino-acids and also buffer substance in the form of phosphate salts and higher nitrogen complexes (proteoses, etc.). A peptone

rich in amino-acids will, of course, furnish a basis for abundant growth. Unless, however, there is an effective buffer for regulating (suppressing) the hydrogen or hydroxyl ion, growth will be rapid at first but will soon cease. This is the experience with the Difco peptone. This peptone is richer in amino-acids (Formol titration) than Witte's peptone but is poorer in buffer (as can be seen from the character of the titration curve—small increases in acid or alkali produce appreciable changes in the P_H.) This latter condition is explicable on the basis of the high initial P_H, which may cause a precipitation of the proteoses at some stage of the preparation. This also accounts for the fact that, although richer in amino-acids, this peptone has the same total nitrogen content as Witte's peptone. It is to this difference in the content of the higher nitrogenous complexes that we ought to look for an explanation of the failure to obtain a potent diphtheria toxin with the Difco peptone.[1]

Realizing that the precipitate at P_H 8.2+ is mostly phosphates its relation to the character of the medium is quite apparent. Phosphate salts play a very important rôle in regulating bacterial metabolism and act with the proteoses as buffers in the medium. The point at which precipitation occurs is near the turning-point of phenolphthalein, the reaction of all sugar media. This explains the variability obtained in the growth of streptococci on such media, as well as the failure to obtain growth of tubercle bacilli in glycerol media made up in that way, even if the reaction is brought back to +1.0. In both cases undoubtedly the partial removal of the phosphate salt is, in itself, a factor. In the former, however, the suppression of the buffer action may be equally important.

[1] The accepted view is that diphtheria toxin is a secretion by the cell of a complex built up by it. This conception it seems to me is not supported by the facts. The more plausible idea is that it is an excretion of a residue in the cell metabolism just as ammonia or lactic acid is a waste by-product. This residue is only produced from the higher peptone complexes. That is why the Difco peptone, poor in these compounds, is not favorable for toxin production. This view also accounts for the failure on the part of *Corynebacterium diphtheriae* to produce toxin in sugar media. The sparing action of sugar on protein or rather nitrogenous metabolism is well-known.

OBSERVATIONS ON THE TYPES OF ORGANISMS ISOLATED FROM WATER AFTER TREATMENT WITH CALCIUM HYPOCHLORITE

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The hypochlorite treatment of public water supplies has within the past few years received a decided impetus. As early as 1888 the late Thomas M. Drown recommended its use, but not until 1908 did it receive serious consideration (Harrington, 1911).

The method generally employed at the present time is the addition of chlorinated lime, popularly known as "chloride of lime" or "bleaching powder," in sufficient amounts to destroy harmful bacteria and render the water potable, but not to interfere with its palatability.

The amount added is expressed in terms of "available chlorine," as determined by the usual thiosulphate titration. In reality this represents the oxygen liberated by chlorine. The reaction is expressed as follows:

$$Ca\begin{cases} O\,Cl \\ Cl \end{cases} + CO_2 = Ca\,CO_3 + Cl_2$$

$$2\,Cl + H_2O = 2\,H\,Cl + O$$

The chlorine liberated unites with the hydrogen of the water to form hydrochloric acid, thus liberating the oxygen which accomplishes the destruction of the bacteria.

According to Clark and Gage (Rosenau, 1916) the water content before treatment is an important factor in determining the amount of chlorinated lime necessary. They found that 0.1

part of available chlorine per 100,000 parts of water effected a satisfactory purification of the Merrimac River with results equal to those of slow sand filtration. *B. coli* was entirely eliminated. In Pittsburgh it was found that 0.13 parts of chlorine per 1,000,000 parts of water were sufficient to sterilize the Alleghany River water after it had passed through the sand filters. It required, however, as much as 1 part per 1,000,000 to accomplish the same results in the raw water.

The following study was made, under the direction of Prof. C.-E. A. Winslow, in order to determine the nature of the bacteria resisting the action of chlorine in such quantities. Croton water, as supplied to New York City, is purified by the addition of calcium hypochlorite in the proportion of 1 part of available chlorin to 2,000,000 parts of water. It was decided to use this water for the experiments. On learning, however, that after treatment it passed through an open reservoir before reaching the lower end of Manhattan where the tests were made, and consequently might receive a fresh quota of saprophytic organisms, it was decided to subject it for a second time to the same treatment.

Accordingly, 3 grams of calcium hypochlorite were added to 100 liters of water (1 part available chlorine to 2,000,000 parts of water). The flasks were tightly corked, thoroughly shaken and allowed to stand two hours. At the end of that time six agar poured plates were made, each inoculated with 1 cc. of the treated water. The plates were kept for forty-eight hours at 20°C. At the end of that time one hundred and five colonies were fished. Those of most diverse appearance were selected, although, as will appear later, the majority belonged to a single group.

The cultures were examined for:

Gelatin liquefaction. The amount of gelatin liquefaction was determined in the following manner: 3 cc. of 10 per cent gelatin was placed in tubes 12 mm. in diameter, solidified, and smeared on the top with a small loop of the culture to be tested. At the end of thirty days the depth of liquefaction was measured and recorded in millimeters.

Chromogenesis. The agar cultures used to determine chromogenesis were kept at 20°C. for two weeks. A fixed portion of the growth was removed with a loop and spread evenly on white filter paper. After drying at room temperature, the color was compared with the Winslow color chart (Winslow, 1908).

Acid production in glucose peptone water. For this test Chester's (1904) method was used. Tubes containing 1 per cent glucose peptone water, made 0.8 per cent acid to phenolphthalein, were inoculated and at the end of ten days were titrated.

Bactericidal action of gentian violet. According to Churchman's method, an ordinary Petri dish was divided into two compartments by the insertion of a metal strip a little longer than the diameter of the plate. Plain agar was poured into one compartment and into the other agar plus gentian violet (dilution 1 to 100,000). In making the stroke inoculation, a loop of the broth culture was gently rubbed back and forth a number of times on the agar. The platinum needle was then sterilized and a second loop similarly applied to the gentian violet side.

Tests were also made for acid production and coagulation in litmus milk, indol production, the reduction of nitrates to nitrites, morphology and spore formation.

The following table gives the numerical results obtained:

	MORPHOLOGY		SPORE FORMATION		GELATIN LIQUEFACTION		REACTION IN LITMUS MILK	
	Bacilli	Cocci	Positive	Negative	Positive	Negative	Positive	Negative
Number of strains	100	5	89	16	68	37	98	7
Per cent..........	95.2	4.7	84.7	15.2	64.7	35.2	93.3	6.6

	INDOL PRODUCTION		ACID PRODUCTION IN GLUCOSE		REDUCTION OF NITRATES		INHIBITION BY GENTIAN VIOLET	
	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Number of strains	75	30	61	44	40	65	98	7
Per cent..........	71.4	28.5	58	41.9	38	61.9	93.3	6.6

It is of interest to note that none of the cocci were affected by the gentian violet, while the growth of all the bacilli, save two, was inhibited.

The organisms readily arranged themselves into five groups. *B. subtilis* far outnumbered all the others, sixty-six (62.8 per cent) belonging to that group alone.

Group 1. Rods with endospores—gelatin liquefied, 66 organisms.

Group 2. Rods with endospores—gelatin not liquefied, 23 organisms.

Group 3. Rods without endospores—gelatin not liquefied, 9 organisms.

Group 4. Rods without endospores—gelatin liquefied, 2 organisms.

Group 5. Cocci, without endospores—gelatin not liquefied, 5 organisms.

Four of the groups showed distinct subdivisions. The chart on the following page shows the relationship of the various members.

One organism (group 5–C) reacted in every way identically with those classified as *M. luteus* except that in peptone cultures it gave a decided indol reaction. According to Winslow, this property is very rare. Chester describes three coccus forms possessing the power of indol production in a slight degree, but they are also gelatin liquefiers.

CONCLUSIONS

The organisms found were apparently of the common saprophytic type usually found in air and water. No intestinal forms appeared to survive the treatment in the amount examined. It would appear, therefore, that available chlorine in the proportion of 1 part to 2,000,000 is sufficient to purify surface water obtained under conditions similar to that of the Croton supply.

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Group		Morphology	Spores	Gelatin stab	Amount of gelatin liquefaction	Reaction in litmus milk	Indol	Acid production from glucose	Reduction of nitrates	Inhibition by gentian violet	Chromogenesis	Number of organisms	Probably identical with
					mm.								
1	A	Bacilli	+	Filiform	5–20	Coagulated	+	+	+	+	Cadmium yellow to orange	36	*B. subtilis*—Cohn
	B	Bacilli	+	Filiform	10	Coagulated after 10 days	–	–	+	+	Cadmium yellow	2	*B. simplex*—Chester (1904)
	C	Bacilli	+	Filiform	10–20	Coagulated	+	+	–	+	Cadmium yellow to orange	15	*B. tumescens*—Chester
	D	Bacilli	+	Filiform	5–12	Coagulated	–	–	–	+	Cadmium yellow to orange	13	*B. ruminatus*—Chester
2	A	Bacilli	+	Filiform	–	Coagulated after 10 days	+	+	–	+	Cadmium yellow to orange	21	*B. cuticularis*—Tatoroff
	B	Bacilli	+	Filiform	–	Alkaline not coagulated	–	–	–	–	Light yellow	1	*B. ginglymus*—Ravenel
	C	Bacilli	+	Filiform	–	Alkaline not coagulated	–	–	+	–	Orange yellow	1	*B. cinctus*—Ravenel
3	A	Bacilli	–	Filiform	–	Coagulated after 10 days	–	–	–	+	Medium rose	3	*B. rubescens*
	B	Bacilli	–	Filiform	–	Coagulated after 10 days	±	–	–	+	Light cadmium yellow	6	*B. subflavus*
4		Bacilli	–	Filiform	10	No change	–	–	–	+	Light cadmium yellow	2	*B. convolutum*—Wright
5	A	Coccus	–	Beaded	–	No change	–	–	+	–	Cadmium orange	1	*Staphylococcus mollis*—Dyar
	B	Cocci	–	Beaded	–	Acid not coagulated	–	–	–	–	Light yellow	3	*M. luteus*
	C	Coccus	–	Beaded	–	Acid not coagulated	+	–	–	–	Light yellow	1	*M. luteus?*

THE MORPHOLOGY OF A STRAIN OF B. DIPHTHERIAE

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Those engaged in the production of diphtheria antitoxin have for several years been confronted with considerable difficulty in producing potent toxins. In my own experience the potency abruptly diminished about five years ago, and during subsequent years decreased in such measure as to become alarming. Intermittently, without known cause, potent toxins again resulted, but could not be produced with the same regularity as formerly, and this in spite of the fact that the organisms seemed to grow well, if film formation is taken as indication of successful proliferation.

The strain I have worked with, and which I believe is the one most commonly used, is known as Park no. 8. This strain readily forms a film on beef, veal or horse meat broth, but the nature of the film varies greatly, and in many cases those who have had experience in this line of work can foretell from the nature of the film whether a potent toxin is formed or not. Sometimes a heavy film that is thick, rough and of yellowish-brown color forms, and in these cases toxin formation is usually low. On the other hand, a friable film of a greyish tint and frequently not as heavy as the first-mentioned kind will form a potent toxin.

In casting about for a reasonable cause of the great differences in toxin production of the same strain when grown in broths made apparently alike, I have prepared many stains, primarily with the object of detecting contaminations. I was much surprised to find in many stains that the typical bacillary forms of diphtheria bacilli had disappeared and that coccus forms appeared in their place. The cocci did not appear as well-rounded forms resembling

typical staphylococci, but might easily be mistaken for streptococci in diplococcus form or in short chains. Intermediate forms were also frequently observed. These still retained the outline of a bacillus that seemed to be swollen, then broken up into cocci of various shape and size. Not infrequently diplococci reminding one of the meningococcus were seen.

That these coccus forms were variations of the original bacillary sypes was easily proved. From Löffler's blood serum, made from horse's blood, typical diphtheria bacilli always appeared. Club-shaped forms, granular bacilli, solidly stained fine bacilli in typical parallel arrangement could always be observed. By transferring the blood serum culture to a medium made of veal broth with addition of 0.2 per cent glucose and 1.5 per cent agar a luxurious growth appeared, but the stain showed the coccus forms described. When from a veal-glucose-agar culture the growth was transferred back to blood serum the original bacillary form reappeared after twenty-four hours incubation. After plating from a veal broth culture on veal-glucose-agar the colonies were all formed of diphtheria bacilli, no contamination being present. Transfers to slanted veal-glucose-agar invariably produced the coccus form, while transfers from these slants or from colonies from the plates to blood serum produced the typical bacillary form.

Since this manuscript was written a paper by Mellon (1917) came to my attention. In this paper the author describes the appearance of coccus forms of diphtheroid bacilli which he studied. The reproductions in this paper show forms exactly similar to those which I have observed. However, the author states that his diphtheroid bacilli produced a toxin distinctly different from the toxin produced by true diphtheria bacilli. My coccus-like growths produced toxins that were identical with the true diphtheria toxin, although of relatively low potency. These toxins have been used successfully for the production of diphtheria antitoxin in horses and have produced typical lesions in guinea pigs.

The observations which I have briefly described and those recorded by Mellon seem to indicate a close morphologic rela-

tionship between the members of the *B. diphtheriae* group. An exhaustive study of this relationship might lead to important scientific results and might be of practical value for the detection of diphtheria carriers, inasmuch as abnormal forms which at present lead to negative diagnoses might, when properly interpreted, show the presence of virulent diphtheria bacilli in unsuspected cases.

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NOTE ON THE PREPARATION OF HYPHOMYCETES FOR MICROSCOPICAL EXAMINATION

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While excellent preparations of bacteria can be made by the familiar smear methods, every one who has worked with hyphomycetes realizes that they do not readily yield themselves to the same treatment. The densely felted mycelium does not smear well; only a few fragments remain on the slide; the fruit heads, if present, are ruptured, and the spores liberated. As a result the most satisfactory preparations of hyphomycetes obtainable by the smear method consist of a few hyphal shreads over which are scattered and lodged the liberated spores. The final preparation has but little value for a demonstration of morphological structures and relationships. This is clearly shown in the accompanying photomicrograph of a smear from a colony of *Sporotrichum* (fig. 1). As a result morphological observations are at present largely made from colonies in hanging drops, which from their nature can be but temporary preparations.

In working with a few species of the dermal hyphomycetes, it appeared as if these difficulties might be avoided if agar colonies were sectioned instead of being smeared. Typical surface colonies of a convenient size were removed from Petri dish agar cultures. In the process of removal a sharpened wire was drawn around the colony a short distance from the longest radiating mycelial threads, cutting through the entire thickness of the agar. A wire was then introduced into the agar block from the side, and it, together with the colony, removed from the Petri dish and placed in 10 per cent formalin for fixation.

After twenty-four hours fixation the colonies were removed for imbedding in celloidin in the same manner as blocks of tissue,

namely transferring successively after twenty-four hours immersion in each, from 60 per cent to 95 per cent to 100 per cent alcohol, to absolute alcohol and ether, to thin celloidin and to thick celloidin, after which the agar block containing the colony was mounted and sectioned. We have found that

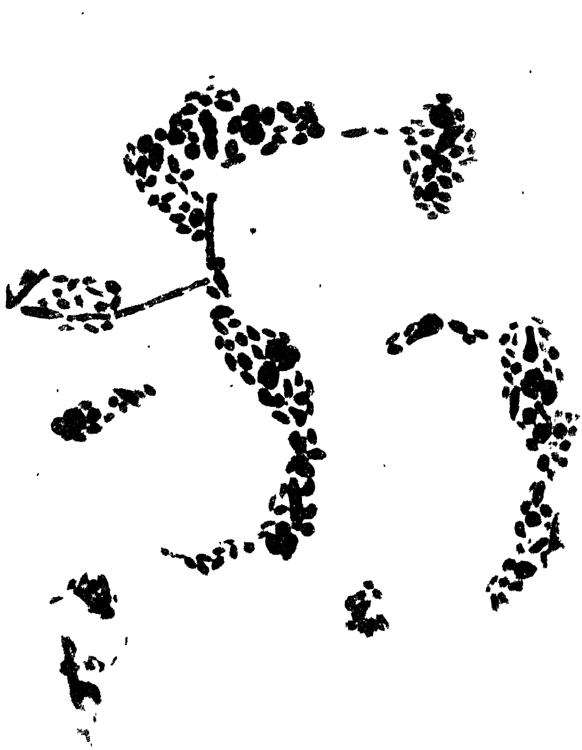

Fig. 1. Smear from Sporotrichum Colony. Zeiss Obj. 2 mm.

hematoxylin gives a more satisfactory stain than the stains ordinarily used for hyphomycetes in smear preparations. After staining the sections were decolorized, dehydrated, cleared and mounted in xylol. Colonies have been sectioned in either horizontal or vertical planes.

In preparations of this character the morphology of the various structures can be studied in their natural relationship to each other. The hyphal felting may be dense in the central portion of the colony, but in the periphery will be found radiating hyphae lying well separated, all sporangia being uninjured.

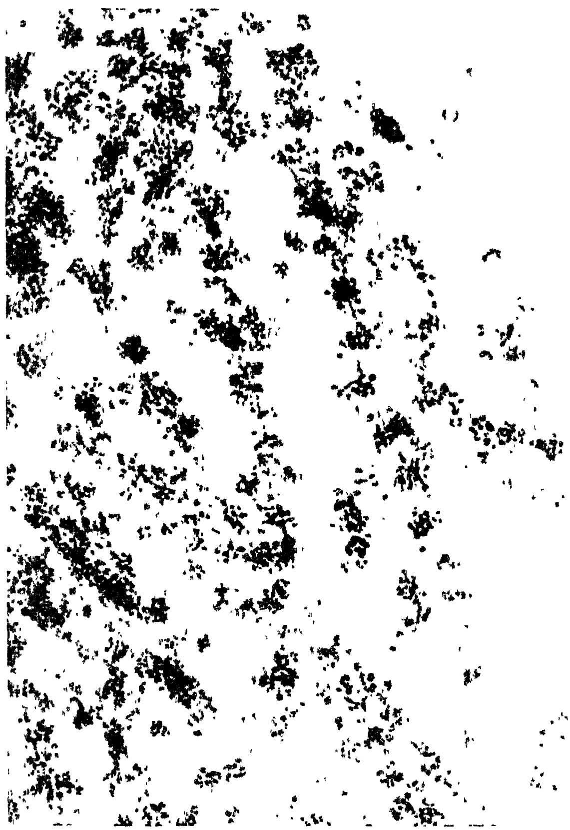

Fig. 2. Section of Sporotrichum Colony. Zeiss Obj. 4 mm.

Furthermore, the preparations are permanent mounts. Figure 2 shows a photomicrograph of a field in a vertical section of an agar colony of *Sporotrichum*. In this instance it is to be noted that the method has preserved the natural grouping of the sporangia about the hyphal threads, and that the morphology is as

clearly discernible as in a hanging drop preparation. The distorted wreckage of the smear preparation is wholly avoided.

The photomicrograhs were made by Mr. Johannes Anderson, technician of the department.

BLASTOCYSTIS HOMINIS: ITS CHARACTERISTICS AND ITS PREVALENCE IN INTESTINAL CONTENT AND FECES IN SOUTH CAROLINA

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Alexeieff (1911) gave the name *Blastocystis enterocola* to a peculiar cell found in the intestines of certain vertebrates (man, guinea pig, rat, chicken and frog) and in the snail (*Haemopsis sanguisuga*), a structure which had previously drawn the attention of numerous observers. He classified with the Ascomycetes.

Ucke (1907), Bohne and Prowazek (1908), and Bensen (1910) had described this cell as an encystment of *Trichomonas intestinalis*.

Alexeieff's contention that it could not be an encysted trichomonas but was in reality a vegetable cell was based on the mucilaginous envelope similar to the capsule of certain Blastomycetes, the observed process of budding, the presence of germinating spores, and the occurrence of the same special structure in the seeds of a certain yeast (*Schizosaccharomyces actosporus*).

Brumpt (1912), who gave the name *Blastocystis hominis* to the species found in man, and Wenyon (1915) agree with Alexeieff as to its nature.

With the view that this cell cannot be a cyst of *Trichomonas intestinalis* I am in full accord. I have seen it often in human feces where no trichomonas could be found and also in the non-trichomonas-infected intestine of man and of the rat, from examination at autopsy of bodies which had not been dead long

enough to allow the trichomonas to disappear had it been present. Then too the true cyst of *Trichomonas intestinalis* which I have described (Lynch, 1916) bears no resemblance to *Blastocystis enterocola.*

Alexeieff has studied the organism in man, the rat, frog and a snail. Wenyon has found it to be very common in soldiers returning from Gallipoli to England.

As to its presence in the United States I have been unable to find any record. It is not mentioned in various books dealing with the examination of feces in this country. I am unable to find any reference to its occurrence in our various indices to medical literature. Siler and Nichols (1911) make no mention of it in their reports of examinations of the feces of one hundred and thirteen pellagrins and two hundred and sixty-nine patients ("practically all non-pellagrous") at Peoria, Kankakee, and Dunning, Illinois. Roberts (1913) in summarizing various reports of intestinal parasites found in pellagra in America, Italy and Egypt, does not mention it. Ridlon (1916) does not record any observation of it in his study of the feces of ninety-five pellagrins in Savannah. Stiles (1915) records no observation of it in an examination of twelve hundred and eighty-seven school children for animal intestinal parasites. Rosenberger (1911) makes no mention of it in his examination of twelve hundred and eighty patients for intestinal parasites in Philadelphia, although he very kindly aided me in my first recognition of the organism and assures me that he has seen it a number of times.

Of course reports of special studies of the prevalence of animal parasites may not be expected to record the occurrence of this organism which is regarded as a vegetable cell, but from my experience in South Carolina I feel sure that it is fairly common in this country, and that the general lack of any record of its occurrence is largely due to failure to recognize it.

In the examination of forty-five pellagrins in Charleston, I have encountered the organism in twenty-five cases, or in 55.5 per cent; in company with *Endamoeba coli* in thirteen of these, and with *Trichomonas intestinalis* in eleven, while all three of

these parasites were present in six of the cases. From the examination of the feces of one hundred and sixty-eight non-pellagrous patients in the same hospital, I found sixty-eight, or 40.4 per cent, to harbor the organism, in company with *Endamoeba coli* in thirty-two, with *Trichomonas intestinalis* in twenty, and associated with both of these protozoa in sixteen of the cases.

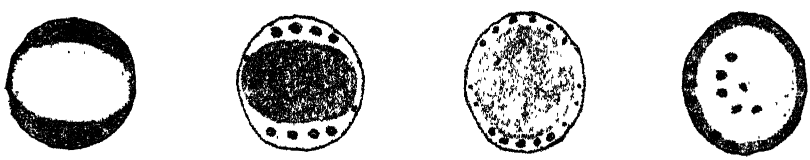
From a similar study of the actively pellagrous inmates of the South Carolina State Hospital for the Insane at Columbia, I found the organism present in seven of twenty patients, or 35 per cent, while in the old pellagrins without symptoms of the disease it occurred in three of twenty-seven cases, or in 11.1 per cent.

In the intestine of twenty-five cases of pellagra autopsied while the body was still warm in the Roper Hospital of Charleston the organism was constantly present.

In examination of feces it has occurred in greater numbers and of larger size in those with diarrhoea, and while it has been found in formed stools it occurs in larger numbers when a purge is given to those without diarrhoea. In the postmortem examinations it has been found to be limited to the large intestine and to the cecum where it is present in large numbers; when the pellagrous inflammation has extended into the lower end of the ileum the Blastocystis has accompanied it, but has gone no further. In the intestines the number of these organisms has appeared to vary directly with the grade of inflammation present. Control examinations of the intestine of the non-pellagrous have shown it to be present in about the same proportion as in feces, and also to be limited to the large intestine.

In feces the cell conforms very well to the characteristic appearance as described by Alexeieff and by Wenyon. It varies in size from about 5 to 15 microns and is generally rounded, though elongated forms are seen. It has a delicate capsule, which apparently allows some alteration in shape, enclosing a rim of cytoplasm which may be uniform in thickness in the rounded cells or thin in the midline and broad at each end in the elongated cells. A large central body or vacuole fills the major

part of the cell. Wenyon describes the rim of cytoplasm as being of greenish appearance and containing nuclei of the appearance of greenish refractive spots, and says that the great part of the content is a large vacuole. While this appearance is commonly observed, it is just as common to find the rim of cytoplasm clear or transparent, containing highly refractive nuclear bodies, and the major portion composed of a large central body of pale hyaline greenish yellow appearance. In other words the organism apparently reverses the materials of the content of its two internal parts (see figs. 1, 2, 3 and 4). This phenomenon has been observed to occur in the course of a few minutes. A cell with the hyaline greenish yellow rim and cen-



1 2 3 4

FIGS. 1, 2, 3, 4. FRESH UNSTAINED SPECIMENS OF BLASTOCYSTIS HOMINIS FROM HUMAN FECES. COMMON FORMS SHOWING REVERSAL OF APPEARANCE OF INTERIOR

tral transparent body changed slowly to one with a transparent rim and a central body of the same appearance as was the rim previously.

In addition to this change, which may be from either form to the other, the cell has been observed to change shape (see fig. 5), passing from a rounded to an elongate form and also becoming curved while being watched for a short while. The change in shape does not appear to alter the internal characteristics.

Large rounded forms have also been seen to extrude through the capsule a large sac of transparent material, the wall and content of which was continuous with that of the central vacuole (see figs. 6, 7, 8 and 9). This sac was projected at the midline

on one side where the cytoplasmic rim was thin. It had a broad neck at first, but this gradually became constricted until the sac was turned loose and the cell became closed again. The extruded sac soon faded from view. The extrusion was as rapid as the throwing out of material from an encysting endamoeba, but the constriction and cutting loose of the sac was very gradual.

These phenomena, the reversal of internal appearances, the change in shape, and the extrusion of material from the central vacuole, have been observed only in the very fresh specimens which were kept at body temperature.

Binary division has been observed by keeping a cover glass preparation at body temperature for several days The cell divided equally until two and four were arranged in rectangular group, and then these disappeared. Stages of this division may be seen in fresh specimens from feces and intestine. Elongated forms, constricting forms and rectangular groups of two, four (see figs. 10, 11, 12 and 13) and sometimes eight cells are not uncommon.

These various phenomena have been observed chiefly in the stools of patients with diarrhoea or in specimens taken directly from the upper large intestine. In formed stools and in older specimens the small round type is all that is usually seen.

It has been interesting to observe the increase in size as well as in number from dysenteric patients, and particularly from the inflamed colon of pellagra. In formed stools from apparently normal intestines the number is small and the cells average about 8 or 9 microns in diameter. In dysenteric stools the number may be enormous and the size is larger, the cell diameter usually being around 15 microns, although many small forms may be present. In the inflamed cecum of pellagra the number is commonly large and the cells vary in size from 5 to 25 or more microns, many being very large. Especially is this true when they are present in ulcers. The exact meaning of this cannot, of course, be definitely stated until we know the properties of the organism. It appears that, while it does not propagate in the normal intestine and its contents to such an extent as to produce large numbers in feces, certain morbid processes of the large

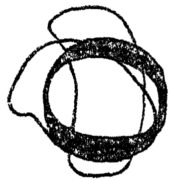
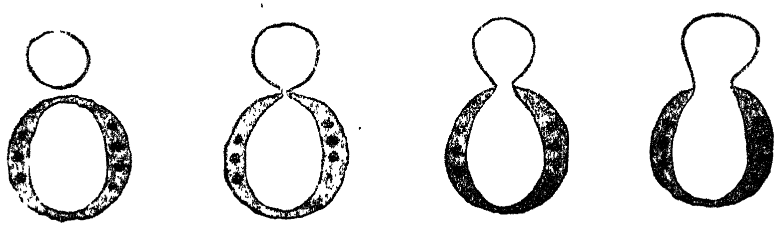
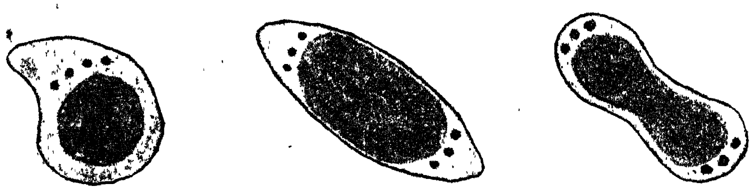


FIG. 5. CHANGE IN SHAPE OF BLASTOCYSTIS HOMINIS IN HUMAN FECES. FRESH SPECIMEN



6 7 8 9

FIGS. 6, 7, 8 AND 9. EXTRUSION OF MATERIAL FROM CENTRAL VACUOLE BY BLASTOCYSTIS HOMINIS IN HUMAN FECES



10 11 12

FIGS. 10, 11 AND 12. FRESH UNSTAINED SPECIMENS OF BLASTOCYSTIS HOMINIS FROM HUMAN FECES. ELONGATING FORMS

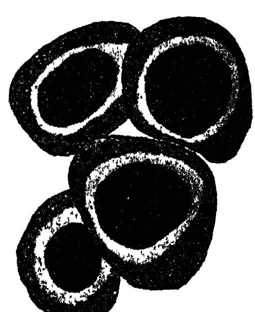


FIG. 13. GROUP OF BLASTOCYSTIS HOMINIS FROM CECUM IN CASE OF PELLAGRA AT AUTOPSY. HEIDENHAIN'S IRON-HAEMATOXYLIN AND EOSIN STAIN

gut offer a better field, and it increases in number and also in size, while in case of diarrhoea large numbers and also many large vegetative forms may be thrown out. It appears that the pellagrous intestine is a splendid field for the growth of this cell. as it also appears to be for small yeasts, since they are usually abundant. The organism is not so common and is of smaller size in the feces of old pellagrins in whom the symptoms of the disease have disappeared.

It has also been interesting to note the differences in viability of the organism from different sources. In all specimens it has disappeared in the course of a few days, and in some exposure for a few hours leads to its elimination. It seems that in the formed stool, where the small rounded form is present, it lasts longest, while in the diarrhoeal stool or in the specimen from the inflamed cecum, where large cells showing the various active phenomena previously described are seen, it does not survive long. Apparently this small rounded type is the resistant stage of the organism, and the large active forms are vegetative non-resistant stages. While examples of binary division are commonly observed, I have not seen the process of budding described by Alexeieff.

As to the liability of the uninitiated to confuse *Blastocystis enterocola* with cysts of the endamoebae and trichomonas, I must agree with other writers, since at one time I looked upon it and described it as probably a cyst of *Trichomonas intestinalis*. However, there are distinct points of difference which if known cannot fail to clear up the difficulty. The typical pear shape, the nearly constant size (about 6 by 8 microns), the nucleus and the peculiar internal structures of the cyst of *Trichomonas intestinalis*, should make the recognition of this body not difficult. As between endamoeba and Blastocystis, the cysts of the former are also of fairly constant size, are round, have a thick capsule, the body being uniform and the nuclei, especially in stained specimens, characteristic; while the Blastocystis may vary from 5 to 20 or more microns in diameter in the same preparation, especially when diarrhoea is present or a purgative has been given, it may show rounded and elongated forms, and

may be single or in rectangular groups of two, four or eight. It also has a thin flimsy capsule, and a body composed of two parts, a cytoplasmic rim which may be transparent or of a pale hyaline greenish yellow, uniform in thickness in the rounded cells, but thin in the midline and thicker at the poles in the elongated cells, containing the highly refractive nuclear bodies commonly in groups in the broad portion at the poles, and a large central body which may be transparent or of pale hyaline greenish yellow.

In specimens stained with Leishman's modification of Wright's method the Blastocystis either has a bluish central body and pink cytoplasmic rim with reddish nuclear bodies or a pink central body and a bluish rim with red nuclear bodies, depending on the stage of the cell. It takes the Heidenhain iron-haematoxylin stain very well (see fig. 10), the rim staining pink, when counterstained with eosin, with black nuclear bodies and the central body appearing as a dark smoky or black body within a large clear vacuole. Apparently in the fixing process, this central body shrinks away from its wall leaving an empty space.

As to the exact nature of the cell, I feel that there is more to be learned. It is true that it bears strong resemblance to yeasts. I have, however, been unable to cultivate the organism in various media including those specially designed for the growth of yeasts, and, in so far as I have been able to learn, this has not been accomplished. Then too, the reversal of the appearance of the materials of the central body and cytoplasmic rim, and the extrusion of material from the central vacuole, are phenomena that I am unacquainted with in the yeasts; and for a yeast body it is singularly non-resistant, especially in the large active form.

As to the properties of *Blastocystis enterocola,* other than its morphology, we know nothing. Therefore, the full significance of its presence cannot be judged. I have seen it in the intestine of rats, and it has presented itself to me as a common intestinal parasite of man to which no attention has been paid in this country. Because of the liability of the observer to confuse it

with other encysted intestinal parasites, and since it has appeared to me to be possibly comparable to endamoeba, trichomonas, lamblia, etc., as an indicator of the contamination of the food or drink of an individual or a community with fecal matter of man or of certain lower animals, I consider it of sufficient importance to warrant a report of these observations.

As an intestinal parasite, of the importance of which we know little or nothing, it should receive our serious consideration.

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CHARACTERISTICS OF COLI-LIKE MICROORGANISMS FROM THE SOIL

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I. ISOLATION OF ORGANISMS AND PRELIMINARY OBSERVATIONS

It has been generally believed that all of the aerobic, non-spore forming, lactose-fermenting bacteria are essentially of fecal origin. Prescott and Papasotiriu found similar organisms on grains, but it appears from the very careful and painstaking studies of Rogers and his associates of the United States Department of Agriculture that the grain types may be differentiated from the fecal organisms. They demonstrated that the grain strains decomposed glucose with the liberation of two or more times as much CO_2 as H_2, whereas the bovine fecal strains formed approximately equal volumes of these gases. Later Clark and Lubs showed that in appropriate glucose media the former were alkaline and the latter acid to methyl red. Levine, employing the methyl red and Voges-Proskauer reactions, observed that an alkaline methyl red reaction was correlated with a positive Voges-Proskauer test and that coli-like organisms giving these reactions were not uncommon in sewage, but were presumably absent, or at least very rarely present, in the feces of man, horse, sheep and pig as well as the cow.

The occurrence of these methyl-red negative, Voges-Proskauer positive organisms in sewage coupled with their absence in feces, and the observation of Clemesha, in India, that the incidence of *B. lactis-aerogenes* (a Voges-Proskauer positive organism) increases considerably in lakes and rivers after rainfall, naturally led to the inference that they are essentially soil forms.

Of the previous works on the coli-like bacteria of the soil, those of Houston in England and Konrich in Germany are probably of widest sanitary significance.

Houston examined a number of samples of orchard, garden, pasture and virgin soils including both polluted and unpolluted areas. He concludes that *B. coli* or its "close allies" are very rarely or never found in virgin soils, while they are present in large numbers in other soils especially those which have been contaminated from animal sources.

Konrich examined 547 samples of soil in Germany; 65 per cent showed *B. coli* in from 0.1 to 0.5 gram. He notes that the further away the source was from cultivation the smaller was the proportion of positive results.

In neither of these studies were the organisms isolated differentiated from fecal strains.

The present investigation was undertaken to determine (1) if the methyl-red negative or Voges-Proskauer positive organisms are the predominant coli-like forms in soil, and (2) to study the characteristics of the various types of aerobic lactose-fermenting organisms isolated from soils.

Source of samples. Forty-two samples were studied, including 9 from different parts of a corn field and one from a clover field in Ames, Iowa; 13 from fallow and 11 from cropped experimental plots; 4 from orchards; and 4 miscellaneous samples sent in by the Soils Department from different parts of the state.

Collection of samples. All samples were secured in October and the early part of November, 1915. The eight orchard and miscellaneous samples furnished by the Soils Department were parts of sample regularly collected for chemical analysis. All other samples consisted exclusively of surface earth collected in the following manner. A sterile wide-mouthed bottle was held horizontally upon the ground, the stopper carefully removed, and 50 to 75 grams of the surface soil (usually from the first inch) was scraped into the bottle with a sterile metallic scalpel or spatula. The bottle was then closed securely, labeled, and taken to the laboratory where it was examined within two or three hours after collection. The spatula or scalpel was sterilized, between samples, by dipping in alcohol and flaming.

Technique. Immediately on arrival at the laboratory, the soil was transferred to a liter flask of sterile water, thoroughly shaken, and after standing for fifteen minutes, or long enough to permit sedimentation of the heavier soil particles, various dilutions of the supernatant liquid were plated on litmus lactose agar. Incubation was at 37°C. One cubic centimeter was also inoculated into an anaerobic Durham tube of lactose broth sealed with paraffine oil. If gas was formed after twenty-four to forty-eight hours at the body temperature litmus lactose agar plates were poured. From the plates made directly or after preliminary enrichment, acid colonies were fished into standard lactose and sucrose broth and into 0.5 per cent glucose-peptone-di-potassium-phosphate solution, in Durham fermentation tubes. After three days at 37°C. gas was recorded and also the methyl-red and Voges-Proskauer reactions. For the methyl-red reaction 0.2 cc. of the indicator was added to 5 cc. of the glucose culture. The Voges-Proskauer reaction was determined by adding an equal volume of 10 per cent KOH to 2 or 3 cc. of the glucose medium. After standing six to twenty-four hours an eosine-like coloration developed if the test was positive.

Results of preliminary studies

The results may be conveniently considered on the basis of the source of the samples under the following heads. Samples from (1) corn fields; (2) fallow experimental plots; (3) cropped experimental plots; (4) miscellaneous samples about which no definite or detailed information was available.

Samples from corn fields. Samples A, B, C, D, E, and H were taken from widely separated parts of a corn field while the crop was still standing. Samples XXXIV and XXXV were obtained from the same field six weeks later; the corn was already in shock. Corn had been grown on this field for three years and manure was last applied in 1913 or two years before this study. One sample, XXXVI, was obtained from a field of Kafir corn, but no information as to its soil treatment was obtained.

Lactose-fermenting bacteria were obtained from eight of these nine samples. Sixty-six coli-like organisms were isolated. Of these 61 (92.5 per cent) formed gas from sucrose, 43 (65.2 per cent) were positive for the Voges-Proskauer reaction, while 56 (84.9 per cent) were alkaline to methyl red.

Samples from fallow experimental plots. Thirteen samples (I to XIII) were obtained from fallow experimental plots. These plots had been under the supervision of the Soils Department of the Iowa State College for seven years during which period they were free from weeds and kept absolutely fallow. Data as to their treatment is indicated in table 1. Samples were taken prior to the 1915 treatment.

Coli-like organisms were not obtained from seven samples I, II, III, IV, VI, VII, and IX. Samples VIII and X each yielded a single lactose fermenter. From samples V, XI, XII and XIII, 83 coli-like cultures were obtained.

Of the 85 organisms isolated, 84 (98.8 per cent) formed gas from lactose and 57 (67.1 per cent) from sucrose; 53 (62.3 per cent) were positive for the Voges-Proskauer reaction, and 53 (62.3 per cent) were alkaline to methyl red.

Samples from cropped experimental plots. Eleven samples were taken from experimental plots on which crops were growing. These plots were also under observation of the Soils Department for seven years previous. On plots of samples XIV and XXXII timothy was grown each year and the stubble plowed under. No other treatment was given. The other nine samples XXIII–XXXI) were treated in various ways as indicated in table 1, and corn was raised upon them each year. Samples were collected in October, 1915, while the crops were still standing.

Coli-like organisms were isolated from every sample. Of 166 cultures obtained, 158 (95.2 per cent) gave gas from lactose and 143 (86.2 per cent) from sucrose; 97 (58.4 per cent) were positive for the Voges-Proskauer reaction and 199 (71.8 per cent) alkaline to methyl red.

Miscellaneous samples. In this group are included 9 samples about which no information as to soil treatment was available.

TABLE 1

Source, treatment, etc., of 42 samples of soil studied

SAMPLE	SOURCE	SOIL TREATMENT OR OTHER REMARKS	DATE OF LAST TREATMENT	CROP	NUMBER OF COLI-LIKE ORGANISMS OBTAINED
A	Corn field	Manured, corn grown for three years	1913	Corn	0
B	Corn field	Manured, corn grown for three years	1913	Corn	14
C	Corn field	Manured, corn grown for three years	1913	Corn	4
D	Corn field	Manured, corn grown for three years	1913	Corn	3
E	Corn field	Manured, corn grown for three years	1913	Corn	2
H	Corn field	Manured, corn grown for three years	1913	Corn	9
I	Expt. plot 114	4 tons manure annually	1914	None	0
II	Expt. plot 113	2 tons manure annually	1914	None	0
III	Expt. plot 112	1 ton manure annually	1914	None	0
IV	Expt. plot 111	4 tons of clover chopped and ploughed under annually	1914	None	0
V	Expt. plot 110	2 tons of clover chopped and ploughed under annually	1914	None	18
VI	Expt. plot 109	1 ton of clover as in IV	1914	None	0
VII	Expt. plot 108	2 tons of oat straw chopped and ploughed under annually	1914	None	0
VII	Expt. plot 107	No treatment, check plot	1914	None	1
IX	Expt. plot 106	2 tons of timothy chopped and ploughed under annually	1914	None	0
X	Expt. plot 105	1 ton timothy as in IX	1914	None	1
XI	Expt. plot 104	8 tons of clover once in four years	1913	None	26
XII	Expt. plot 103	8 tons manure once in four years	1913	None	24
XIII	Expt. plot 102	28 tons peat annually	1914	None	14
XIV	Expt. plot 101	Timothy grown, each year and stubble plowed under	1914	Timothy	18
XV	Orchard	Cloved sod		Clover	12

TABLE 1—Continued

SAMPLE	SOURCE	SOIL TREATMENT OR OTHER REMARKS	DATE OF LAST TREATMENT	CROP	NUMBER OF COLI-LIKE ORGANISMS OBTAINED
XVI	Orchard	Cover crop planted in summer and ploughed under in spring		?	0
XVII	Orchard	Clean cultivation			0
XVIII	Orchard	Blue grass sod		Blue grass	8
XIX	Sioux loam	Clover field recently in oats		Clover	1
XX	Wabash silty clay loam	Surface soil			1
XXI	Marshall silt loam	Surface soil			8
XXII	Corrington loam	Surface soil; corn field		Corn	5
XXIII	Expt. plot 213	No treatment, check plot		Corn	13
XXIV	Expt. plot 214	Legume treatment, cow peas in July. Two years out of four in rotation with corn	1913	Corn	18
XXV	Expt. plot 215	8 tons manure	1913	Corn	15
XXVI	Expt. plot 216	8 tons manure with cow peas	1913	Corn	14
XXVII	Expt. plot 217	8 tons manure with 800 pounds bone meal and cow peas	1913	Corn	16
XXVIII	Expt. plot 218	800 pounds bone meal and cow peas	1913	Corn	16
XXIX	Expt. plot 219	8 tons manure and 800 pounds bone meal	1913	Corn	13
XXX	Expt. plot 220	800 pounds bone meal and 200 pounds K in KCl and cow peas	1913	Corn	19
XXXI	Expt. plot 221	8 tons manure, 800 pounds bone meal and 200 pounds K in KCl	1913	Corn	12
XXXII	See XIV	See XIV	1913	Timothy	10
XXXIII	Clover field	Unknown	1913	Clover	13
XXXIV	Corn field	Same as A	1913	Corn	17
XXXV	Corn field	Same as A	1913	Corn	15
XXXVI	Corn field	Unknown	1913	Kafir corn	2

Samples XVI and XVII did not yield any coli-like organisms. From XIX and XX a single culture was recovered in each case. From the five samples remaining, XV, XVIII, XXI, XXII and XXXIII, 46 coli-like organisms were obtained.

Of the 48 strains isolated, 45 (93.8 per cent) formed gas from lactose, and 43 (89.6 per cent) from sucrose; 27 (56.3 per cent) were positive for the Voges-Proskauer reaction and 35 (73 per cent) were alkaline to methyl red.

A comparison of the fallow and cropped experimental plots with respect to the presence of coli-like forms is shown in table 2. It is seen from tables 1 and 2 that, whereas coli-like bacteria

TABLE 2

Presence of coli-like bacteria in experimental soil plots at Ames, Iowa

COLI-LIKE ORGANISMS	FALLOW PLOTS		CROPPED PLOTS	
	Number	Per cent	Number	Per cent
Absent	7	53.8	0	0.0
Rare	2	15.4	0	0.0
Abundant	4	30.8	11	100.0

were only occasionally isolated from fallow plots, some of which had considerable quantities of manure applied at different times to them, the cropped experimental plots invariably showed coli-like bacteria present.

The reactions of the 365 organisms isolated are summarized in table 3. The organisms from the fallow experimental plots attacked sucrose less frequently (67.1 per cent), than those from other sources (86.2 to 92.5 per cent).

These preliminary observations indicate that among the soils studied

1. Coli-like organisms are more abundant in soils bearing crops (corn, clover and timothy) than in fallow soils, even though considerable quantities of manure had been added to the latter.

2. The most common types of coli-like forms found in soil are sucrose fermenters, alkaline to methyl red in glucose media and reacting positively to the Voges-Proskauer test.

TABLE 3

Preliminary observations of 365 coli-like bacteria obtained from soil

	ORGANISMS FROM									
	Corn fields		Fallow experimental plots		Cropped experimental plots		Miscellaneous samples		All samples	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
Voges-Proskauer positive	43	65.2	53	62.3	97	58.4	27	56.3	220	60.2
Methyl-red negative	56	84.9	53	62.3	119	71.8	35	73.0	263	72.1
Gas from:										
Glucose	66	100.0	85	100.0	166	100.0	48	100.0	365	100.0
Lactose	66	100.0	84	98.8	158	95.2	45	93.8	353	99.5
Sucrose	61	92.5	57	67.1	143	86.2	43	89.6	304	83.4

3. Of 365 cultures studied, all forming gas from glucose, 353 (99.5 per cent) gave gas from lactose and 304 (83.4 per cent) from sucrose; 220 (60.2 per cent) were positive for the Voges-Proskauer reaction, and 263 (72.1 per cent) were alkaline to methyl red.

4. The methyl red and Voges-Proskauer reactions seem well correlated. Of 263 alkaline to methyl red 81 per cent gave a positive Voges-Proskauer reaction, while among 220 strains which gave the Voges-Proskauer test 96 per cent were alkaline to methyl red.

II. TYPES OF COLI-LIKE BACTERIA IN THE SOIL

The preceding observations were preliminary in character and some of the cultures were probably not pure. The organisms were therefore re-purified by plating on brilliant green agar containing 1 part in 500,000 of the dye. A very small loop of a twenty-four hour lactose broth culture of the organism to be purified was placed near the center of a Petri dish containing brilliant green agar and smeared over the surface of the medium with a sterile glass rod. A second plate was then smeared with the same rod. The Petri dishes were incubated for forty-

eight hours at 37°C. The first plate often showed extensive over-growths, but in these instances well isolated colonies were almost always present on the second plate. If acid colonies were present, one was fished into lactose broth. If distinctly acid colonies were not present, then a colony which most nearly resembled *B. coli* was fished into lactose broth. The broth tubes were incubated at 37°C. for forty-eight hours and, if gas was formed, agar slants were made from them.

Upon some of the brilliant green agar plates two types of colonies were present. They were similar in shape and often in size, but one was white and heavy while the other showed a light or opalescent thin growth. Where these two kinds of colonies appeared fishings were made from both.

About 400 cultures were obtained, but as these were too many to handle only 184 were studied in detail.

Characters studied

Morphology, Gram's stain, acid production and coagulation in milk, gelatin liquefaction, indol production, motility, the methyl red reaction and production of gas and acetyl-methyl-carbinol from various substances were observed.

Morphology and gram stain. Gram stains were made from young (less than twelve hours old), agar slant cultures. Freshly prepared staining solutions were employed and their reliability determined by staining known positive (*B. subtilis*) and negative (*B. coli*) organisms. Smears were immersed for one minute in anilin oil gentian violet, then for one minute in Gram's iodine solution, decolorized for five minutes in 95 per cent alcohol and counterstained for fifteen seconds with dilute safranin. All of the organisms studied are regarded as Gram-negative, but it was observed that some were not decolorized as completely as others.

Morphology was determined from the Gram stain. Five of the cultures seemed to contain both long and short rods. All of the others (179) were short rods.

Milk. Acid production and coagulation was observed in

azolitmin milk. The reaction of the medium was + 1 to phenolphthalein and incubation was at 37°C. for forty-eight hours. Acid production was recorded as: + acid; sl. slightly acid; ∓ no change or neutral; – alkaline.

Coagulation after forty-eight hours incubation without heating was recorded +. Tubes which did not show coagulation were heated in a boiling water bath for one or two minutes.

Gelatin liquefaction. Stab cultures in nutrient gelatin were observed for thirty-four days at 20°C. Observations were made after 2, 7, 15, 20, 27 and 34 days. The records finally used were for the thirty-four day period.

Motility. Motility was determined from stab cultures in semi-solid nutrient or Hesse agar (5 grams agar per liter) as suggested by Kligler. The medium was kept in the 37° incubator for two or three hours before inoculation. Where motility was not present, growth was confined to the line of inoculation, the rest of the medium remaining perfectly clear. The motile forms quickly grew out away from the line of inoculation in all directions, causing a diffuse clouding in the medium. Very often the growth extended for a distance of 10 or 15 mm. in six hours at 37°C. Observations were made after six and twenty-four hours.

Indol production. The formation of indol from 1 per cent Witte's peptone solution was determined after incubation at 37°C. for four days. The test was made by adding a few drops of 10 per cent H_2SO_4 to about 8 cc. of the peptone culture, and, after shaking the mixture, 1 cc. of a freshly prepared 0.01 per cent $NaNO_2$ solution was added so as to form a layer on the surface. The tubes were set aside for five or six hours after which time the presence or absence of the nitroso-indol coloration at the junction of the two liquids was recorded.

Methyl-red reaction. For the methyl-red reaction the 0.5 per cent peptone di-potassium-phosphate-glucose medium suggested by Clark and Lubs was employed. Incubation was at 37°C. for forty-eight hours, and 0.2 cc. of the indicator was added to 5 cc. of the culture for the test.

Gas and acetyl-methyl carbinol production. The monosaccharids

glucose and galactose, the disaccharids lactose and sucrose, the trisaccharid raffinose, glucoside salicin, the alcohols glycerol, mannitol and dulcitol, and the polysaccharids dextrin, inulin and starch were studied for gas formation and acetyl-methyl-carbinol production. The medium for these reactions consisted of 0.5 per cent di-potassium phosphate peptone solution to which was added the test substance. The following quantities of test materials were employed: glucose, galactose and lactose 0.5 per cent; raffinose, dulcitol and salicin 0.71 per cent; sucrose, mannitol, glycerol, dextrin, inulin and starch 0.75 per cent.

The polysaccharids were incubated for seventy-two hours and the other test substances for forty-eight hours at 37°C.

Gas formation was observed in Durham fermentation tubes. Acetyl-methyl-carbinol was tested for by adding 10 per cent KOH solution to an equal volume of the culture and allowing the mixture to stand exposed to the air. Records were made after five or six and after twenty-four hours. The results were recorded as follows: + gas positive, acetyl-methyl carbinol positive; ± gas positive, acetyl-methyl carbinol negative; – gas negative, acetyl-methyl carbinol negative; ∓ gas negative, acetyl-methyl carbinol positive.

Correlation of results

The correlation of the Voges-Proskauer and methyl-red reactions with these purified cultures was more marked than was obtained in the preliminary tests. Of 139 which gave the Voges-Proskauer reaction 132 (95.0 per cent) were alkaline, 4 (2.9 per cent) were in the neutral tints, and 3 (2.1 per cent) acid to methyl red. Of 134 strains alkaline to methyl red 132 (98.6 per cent) gave the Voges-Proskauer reaction. Of 45 strains which did not give the Voges-Proskauer reaction after forty-eight hours at 37°C. 30 (66.7 per cent) were acid, 13 (28.9 per cent) in the neutral tints and 2 (4.4 per cent) alkaline to methyl red. The two alkaline and 8 of the 13 neutral strains attacked the polysaccharids dextrin and starch and were non-motile, while all of the other Voges-Proskauer negative strains

were motile and did not form gas from the polysaccharids. We believe that on longer incubation (five to seven days) these 8 neutral strains would have reverted to a distinct alkaline reaction, and together with the two alkaline cultures would probably react positively for the Voges-Proskauer test.

In table 4 are shown the number and per cent of positive reactions for 177 organisms which formed gas from lactose. Tests which were uniformly negative or positive are not included in the table. With 7 cultures gas formation from lactose was questionable. These are discussed later in more detail.

TABLE 4

Reactions of aerobic non-sporing lactose-fermenting bacteria in the soil

CHARACTER	NUMBER POSITIVE	PER CENT POSITIVE
Voges-Proskauer	142	80.3
Motility	123	69.6
Gelatin	83	46.8
Indol	66	37.3
Sucrose	165	93.3
Raffinose	162	91.6
Dulcitol	74	41.8
Glycerol	78	43.1
Salicin	159	89.9
Dextrin	82	46.4
Inulin	21	11.9
Starch	57	32.2

From table 4 it is seen that sucrose, raffinose and salicin are almost always attacked, with gas formation, as indicated by 93.3 per cent, 91.5 per cent and 89.9 per cent positive tests respectively. A large proportion, 80.3 per cent, gave a positive Voges-Proskauer reaction, and 69.6 per cent were motile. Gelatin liquefiers were quite common (46.8 per cent), as were also polysaccharid fermenters (dextrin 46.4 per cent, inulin 11.9 per cent and starch 32.2 per cent). Gas was formed from dulcitol by 41.8 per cent; and glycerol by 43.1 per cent. Only 37.3 per cent formed indol.

In general the reactions of the organisms isolated from soil are quite different from those obtained by Levine from various ani-

mal feces and sewage. A comparison of the reactions of the fecal and sewage strains with the soil strains is shown graphically in figure 1.

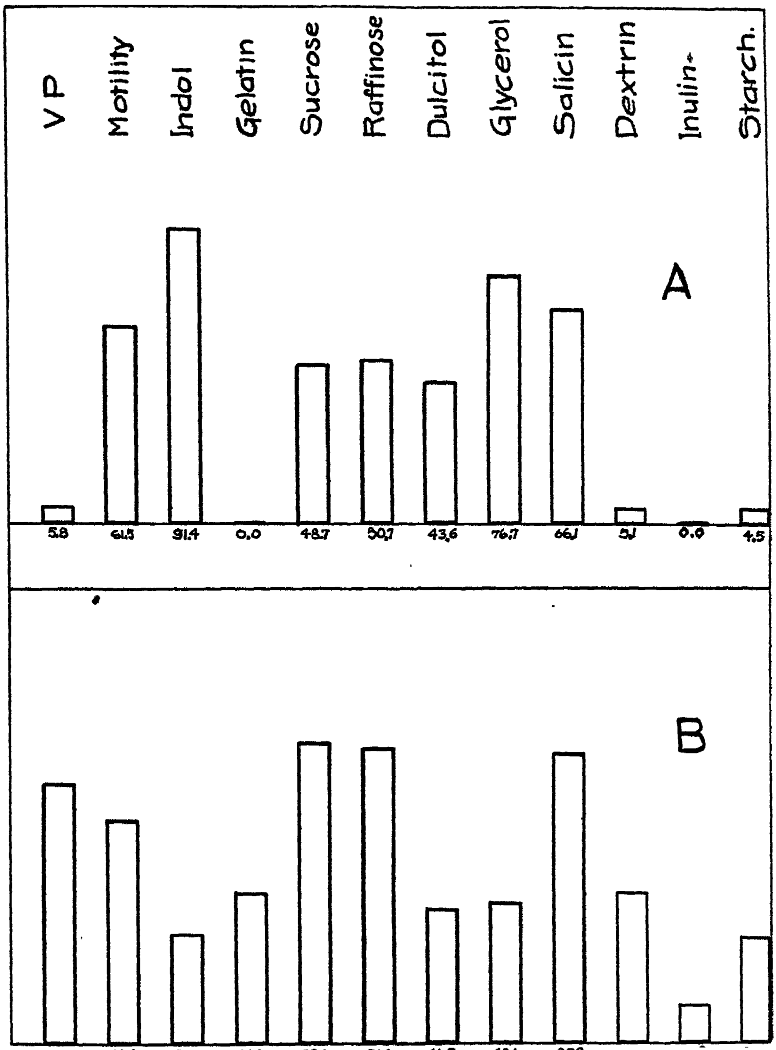

FIG. 1. PER CENT OF POSITIVE REACTIONS OF COLI-LIKE BACTERIA FROM (*A*) FECES AND SEWAGE, AND (*B*) SOIL

There are three distinct and rather well defined groups of aerobic non-spore forming lactose-fermenting bacteria in the soil. The characteristics of these groups, together with that of a small group which we do not feel justified in regarding as distinct for the present, are shown in figure 2 and table 5.

Group A (*B. coli type*). Thirty-five organisms are included in Group A. All are motile, Gram negative, do not form gas from starch nor liquefy gelatin, and react negatively to the Voges-

TABLE 5

Characteristics of the groups of coli-like bacteria obtained from the soil

GROUP		VOGES-PROSKAUER	MOTILITY	GELATIN	INDOL	SUCROSE	RAFFINOSE	DULCITOL	GLYCEROL	SALICIN	DEXTRIN	INULIN	STARCH
A	Number +...	0	35	0	23	26	26	32	17	18	0	0	0
	Per cent +...	0.0	100.0	0.0	65.6	74.3	74.3	91.5	48.6	51.5	0.0	0.0	0.0
B′	Number +...	0	0	0	3	10	10	2	10	10	10	5	10
	Per cent +...	0.0	0.0	0.0	30.0	100.0	100.0	20.0	100.0	100.0	100.0	50.0	100.0
B	Number +...	44	0	2	22	44	44	26	43	44	44	13	43
	Per cent +...	100.0	0.0	45	50.0	100.0	100.0	59.1	97.7	100.0	100.0	29.6	97.7
C	Number +...	88	88	81	15	85	82	14	8	87	28	4	4
	Per cent +...	100.0	100.0	92.1	17.1	96.6	93.2	15.9	9.1	98.9	31.8	4.5	4.5

Proskauer test. Thirty-two are short rods and 3 are presumably mixed as they contained a few long rods as well. Acid was formed in milk by all of the strains and a coagulum was produced in forty-eight hours at 37°C., without heating. Gas was formed as follows: sucrose 74.3 per cent, raffinose 74.3 per cent, dulcitol 91.5 per cent, glycerol 48.6 per cent, and salicin 51.5 per cent. The polysaccharids, dextrin, inulin and starch were not attacked, with gas formation. Indol was formed by 65.6 per cent. In table 6 is shown the correlation of each reaction with all other reactions. Where a reaction was uniformly negative or positive it is not shown in tabular form but indicated by a

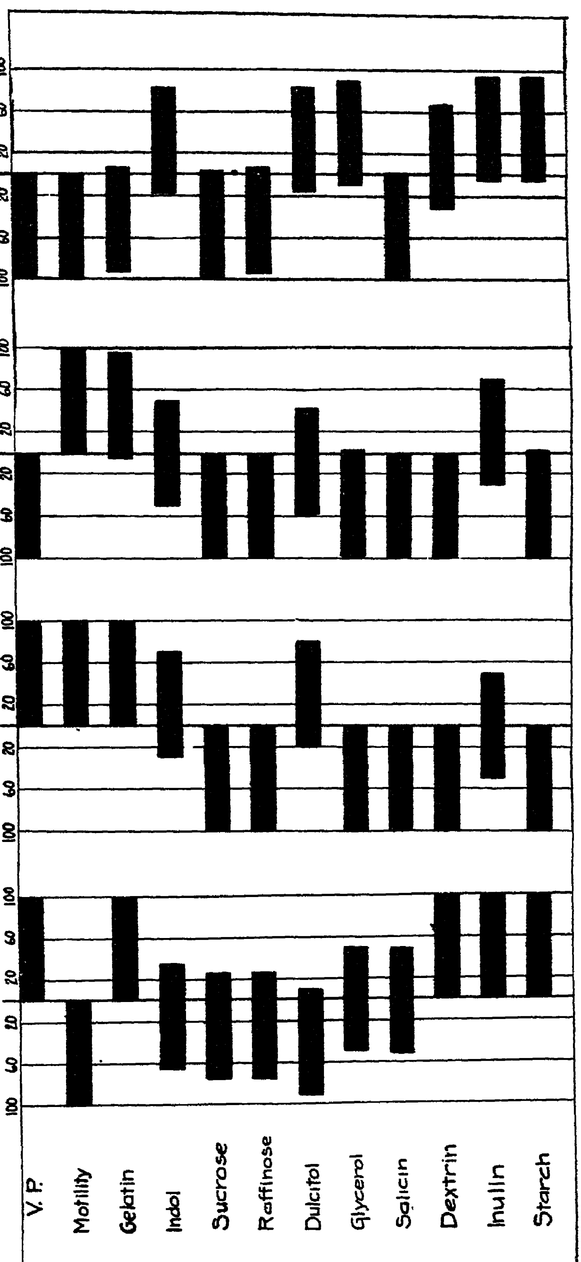

FIG. 2. REACTIONS OF MAIN GROUPS OF COLI-LIKE BACTERIA OBTAINED FROM SOIL

(*A*) *B. Coli* type; (*B* and *B'*) *B. Aerogenes* type; (*C*) *B. cloacal* type

note. It appears from this table and from chart 2 that group A is not a homogenous one, but that it may be further subdivided according to the reaction on sucrose, or perhaps, salicin or glycerol.

Groups B and B′ (B. aerogenes type). Group B includes 44 organisms which give a positive Voges-Proskauer reaction, are non-motile, and practically always form gas from starch. All

TABLE 6

Correlation of reactions in group A (B. coli type) of lactose-fermenting bacteria from the soil

		Indol		Sucrose		Raffinose		Dulcitol		Glycerol		Salicin	
		+	−	+	−	+	−	+	−	+	−	+	−
Indol	+	23		19	4	18	5	23		7	16	14	9
	−		12	7	5	8	4	9	3	10	2	4	8
Sucrose	+	19	7	26		24	2	25	1	12	14	16	10
	−	4	5		9	2	7	7	2	5	4	2	7
Raffinose	+	18	8	24	2	26		25	1	13	13	15	11
	−	5	4	2	7		9	7	2	4	5	3	6
Dulcitol	+	23	9	25	7	25	7	32		14	18	18	14
	−		3	1	2	1	2		3	3			3
Glycerol	+	7	10	12	5	13	4	14	3	17		5	12
	−	16	2	14	4	13	5	18			18	13	5
Salicin	+	14	4	16	2	15	3	18		5	13	18	
	−	9	8	10	7	11	6	14	3	12	5		17

All strains are motile. Gelatin liquefaction, the Voges-Proskauer reaction, and gas formation from dextrin, inulin and starch were in all cases negative.

are gram negative short rods, but one culture also contained some long rods. With respect to the azolitmin milk reaction, it was observed that 8 (18.6 per cent) were only slightly acid after forty-eight hours at the body temperature, while 14 (31.8 per cent) were not coagulated except by heating. Gelatin was liquefied by 2 cultures (4.5 per cent). Positive tests for gas formation were observed as follows: sucrose 100 per cent; raffinose 100 per cent; dulcitol 59.1 per cent; glycerol 97.7 per cent;

salicin 100 per cent; dextrin 100 per cent; inulin (29.6 per cent) and starch 97.7 per cent. Indol was formed by 50 per cent of the strains.

In table 7 is shown the correlation of the various reactions studied. Those uniformly positive or negative are omitted. From this table and chart 2, it appears that subdivision might be made on the basis of indol, dulcitol, or inulin reactions. These reactions, however, are not correlated with each other. Of 22 organisms which formed indol 15 (68.2 per cent) gave gas from dulcitol and 7 (31.8 per cent) from inulin, whereas among an

TABLE 7

Correlation of reactions in group B (B. aerogenes type) of lactose-fermenting bacteria obtained from the soil

		INDOL		DULCITOL		INULIN	
		+	−	+	−	+	−
Indol	+	22		15	7	7	15
	−		22	11	11	6	16
Dulcitol	+	15	11	26		6	20
	−	7	11		18	7	11
Inulin	+	7	6	6	7	13	
	−	15	16	20	11		31

All strains were non-motile; 2 liquefied gelatin; 1 did not form gas from glycerol, and 1 from starch; all formed gas from sucrose, raffinose, salicin, and dextrin.

equal number of indol-negative-strains, 11 (50.0 per cent) gave gas from dulcitol and 6 (27.3 per cent) from inulin. Similarly, there does not seem to be any correlation between gas formation from dulcitol and inulin. 23.1 per cent of the dulcitol-positive and 38.8 per cent of the dulcitol-negative strains gave gas from inulin. We would, therefore, regard the group as a whole as the species *B. aerogenes*, and the sub-groups formed by splitting of inulin, indol or dulcitol as varieties.

Group B' includes ten organisms which did not give the Voges-Proskauer reaction but which attacked the polysaccharids very readily. In these respects the group seems to be intermediate

between A and B. The reaction to methyl red was also inconclusive. Two strains were alkaline and eight in the neutral tints after forty-eight hours at 37°C. As has been previously suggested, we believe that the methyl red reaction would become distinctly alkaline on further incubation, and that the organisms would probably also give the Voges-Proskauer reaction. With respect to motility, indol production, gelatin liquefaction, and gas formation from various fermentable substances, the organisms in Group B′ are so strikingly similar to Group B, and so different from either group, A or C, that it is perhaps best to include them under the species *B. aerogenes*.

Group C (*B. cloacae type*). This group contains 88 organisms and constitutes the predominant form of coli-like microorganisms in the soil. The organisms give the Voges-Proskauer reaction, are motile, and probably always liquefy gelatin. All are Gram-negative short rods. The reaction in azolitmin milk is quite different from that observed in group A. Only 13 cultures (14.8 per cent) of group C were distinctly acid, and 75 (85.2 per cent) were but slightly acid. With 15 strains (17.1 per cent) a coagulum was obtained in the milk without heating; 68 (77.2 per cent) coagulated after heating in a boiling water bath for one or two minutes; with 5 (5.7 per cent) coagulation did not take place even after heating for ten minutes.

A large number of these strains (about 75 per cent) formed only 10 per cent or less gas from lactose. The test for gas formation from lactose was repeated several times in order to verify the fact that the organisms were lactose fermenters. The last observation was made in 1 per cent Witte's peptone containing 0.5 per cent di-potassium phosphate and 1 per cent of Difco lactose guaranteed to be free from glucose. The medium was carefully sterilized for ten minutes at 10 pounds, the pressure quickly released and the medium rapidly cooled by immersion of the tubes in cold water. A very light colored medium was obtained, and it is believed that inversion of the lactose was avoided. The records finally accepted were those made with this medium.

The reactions of group C are as follows:

Gelatin liquefied 92.1 per cent; indol 17.1 per cent; gas from sucrose 96.6 per cent; raffinose 93.2 per cent; dulcitol 15.9 per cent; glycerol 9.1 per cent; salicin 98.9 per cent; dextrin 31.8 per cent; inulin 4.5 per cent and starch 4.5 per cent.

TABLE 8

Correlation of reactions in group C (B. cloacae type) of lactose fermenting bacteria obtained from the soil

		INDOL		SUCROSE		RAFFINOSE		DULCITOL		GLYCEROL		DEXTRIN		INULIN		STARCH	
		+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Indol	+	15		15		15		7	8	6	9	8	7	2	13	2	13
	−		73	70	3	67	6	7	66	2	71	20	53	2	71	2	71
Sucrose	+	15	70	85		81	4	13	72	8	77	28	57	4	81	4	81
	−		3		3	1	2	1	2		3		3		3		3
Raffinose	+	15	67	81	1	82		13	69	7	75	26	56	3	79	4	78
	−		6	4	2		6	1	5	1	5	2	4	1	5		6
Dulcitol	+	7	7	13	1	13	1	14		7	7	6	8	2	12	2	12
	−	8	66	72	2	69	5		74	1	73	22	52	1	73	2	72
Glycerol	+	6	2	8		7	1	7	1	8		5	3	2	6	2	6
	−	9	71	77	3	75	5	7	73		80	23	57	2	78	2	78
Dextrin	+	8	20	28		26	2	6	22	5	23	28		4	24	4	24
	−	7	53	57	3	56	4	8	52	3	57		60		60		60
Inulin	+	2	2	4		3	1	2	1	2	2	4		4		3	1
	−	13	71	81	3	79	5	12	73	6	78	24	60		84	1	83
Starch	+	2	2	4		4		2	2	2	2	4		3	1	4	
	−	13	71	81	3	78	6	12	72	6	78	24	60	1	83		84

All strains are motile and form gas from salicin. Gelatin not liquefied in thirty-four days by 7 strains.

Table 8 shows the correlation of the various reactions. Reference to this table and figure 2 indicates that the group is a rather homogenous and distinct one. Some of the variations observed are probably due to impure cultures, as isolation by the plating method cannot be relied upon always to yield thor-

oughly pure cultures. The characteristics of this group are essentially those of *B. cloacae*.

Kligler observes that gelatin liquefaction is correlated with a negative glycerol fermentation. Rogers, Clark and Evans, however, regarded 80 per cent of gelatin-liquefying lactose-fermenting strains isolated from grains, as glycerol fermenters. This difference, we think, is due to the employment of acid production in place of gas formation as an index of fermentation. Kligler selected 1.5 per cent, and Rogers 1 per cent normal acid as the line of demarcation between fermenters and non-fermenters. If the Kligler standard is applied to the Rogers' strains, only 2 or 3 (5.7–7.5 per cent) are glycerol fermenters, 3 or 4 (7.5–10 per cent) are questionable, while 34 (85.0 per cent) would be regarded as non-fermenters. Acid production as an index of fermentation is particularly unreliable with glycerol. The results obtained in this study, using gas formation as the criterion of fermentation, are well in accord with those of Kligler.

There were included in our series seven organisms which did not form gas from lactose. In all other characters they are like *B. cloacae*. Five of these strains gave a positive test for acetyl-methyl-carbinol from lactose. Of several thousand tests with coli-like organisms, these were the only instances (except one case of a starch reaction) in which a positive reaction for acetyl-methyl-carbinol was not accompanied by gas formation. It is of course possible that some gas was formed, but that the quantity was so small that it was entirely absorbed by the culture medium and therefore not apparent in the closed arm of the fermentation tube.

Differentiation of groups. Briefly stated, we may say that group A differs from B in that it is motile, and does not give the Voges-Proskauer reaction, nor form gas from corn starch. It differs from group C in that gelatin is not liquefied, the Voges-Proskauer reaction is negative, and gas is formed much more commonly from dulcitol and glycerol. The indol reaction is also usually positive for group A but negative for group C.

Group B differs from C in gelatin liquefaction, motility and

gas formation from many substances. Group B is non-motile and rarely liquefies gelatin. C is just the reverse. Gas is practically always formed from glycerol, dextrin and starch by the organisms in group B, but only rarely by those in group C.

Observations on acetyl-methyl-carbinol production from various substances

It was thought that acetyl-methyl-carbinol production from various substances (carbohydrates, alcohols and glucosides) might be an aid in the differentiation of the organisms.

Ferriera, Horta and Paredes indicate that *B. cloacae* differs from *B. aerogenes* by its ability to give the "Proskauer" reaction with galactose and mannitol. Their observations are not confirmed by this study.

Levine pointed out that coli-like forms which did not form acetyl-methyl-carbinol from glucose (Voges-Proskauer strains) rarely formed the carbinol from other fermentable substances, whereas the Voges-Proskauer-positive strains often gave a positive "carbinol" test with other carbohydrates, etc.

Of 45 Voges-Proskauer-negative cultures 2 gave a positive carbinol test with lactose and 1 with starch. With galactose, sucrose, raffinose, dulcitol, mannitol, glycerol, salicin, dextrin, and inulin the reaction was uniformly negative.

Among 139 Voges-Proskauer-positive strains, positive "carbinol" tests were obtained as follows: galactose 89 (64.0 per cent); lactose 83 (59.6 per cent); sucrose 124 (89.3 per cent); raffinose 114 (82.4 per cent); dulcitol 15 (10.8 per cent); mannitol 123 (88.6 per cent); glycerol 6 (4.3 per cent); salicin 94 (6.8 per cent); dextrin 14 (10.0 per cent); and starch 25 (18.0 per cent). Inulin was always negative. The positive reaction with glycerol is particularly interesting because it indicates a synthesis, the acetyl-methyl-carbinol containing one more carbon atom than glycerol.

In a general way, there is very little difference between the groups B and C with respect to formation of acetyl-methyl-carbinol from galactose, sucrose, raffinose, mannitol, and inulin.

Group B reacted positively in 79.6 per cent of the lactose and 56.8 per cent of the salicin tests, as compared with 48.8 per cent and 75.0 per cent respectively for group C. The latter never formed the carbinol from dulcitol and glycerol, and only 1.1 per cent of the strains were positive with dextrin and starch. With group B positive reactions were obtained as follows: dulcitol 34.1 per cent; glycerol 13.6 per cent; dextrin 29.6 per cent and starch 54.6 per cent. It should be noted that gas formation from dulcitol, glycerol and starch shows a much more striking differentiation between *B. cloacae* and *B. aerogenes* than does acetyl-methyl-carbinol production from these substances.

III. CONCLUSIONS

1. It appears that under the conditions prevailing in Ames, Iowa, coli-like bacteria were considerably more abundant in soils upon which crops were growing than in absolutely fallow areas receiving similar soil treatment.

2. The Voges-Proskauer and methyl-red reactions are well correlated.[1]

3. The *aerogenes-cloacae* types, which give a positive Voges-Proskauer reaction and are alkaline to methyl red in Clark and Lubs' peptone-di-potassium-phosphate-glucose solution, are the predominant coli-like forms in soil.

4. The *aerogenes-cloacae* group may be differentiated from the *coli* group by the methyl-red or Voges-Proskauer reaction. *B. aerogenes* differs from *B. cloacae* in that it is non-motile, rarely liquefies gelatin, and forms gas from glycerol and corn starch.

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[1] Since the completion of this paper Greenfield in Kansas, and Hulton in Pennsylvania have confirmed the correlation of the Voges-Proskauer and methyl-red reactions, as observed by Levine.

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THE EFFECT OF STERILIZATION UPON SUGARS IN CULTURE MEDIA

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A great many bacteria possess the power of fermenting carbohydrates. As a rule, a given species is able to ferment more than one kind of carbohydrate, but the various species are sufficiently limited and varied in this power so that they may often be distinguished and identified by studies of their fermentative abilities. To ascertain the fact whether a sugar has actually been fermented, it would, of course, be absolutely decisive to isolate, by chemical analysis, a product characteristic of the sugar in question. The problem, however, can not in general be solved in this way, since the work involved is very great. As a rule, it is sufficient to demonstrate the production of acid or of gas as a result of growth in the medium. Acid or gas production is not characteristic of any particular sugar. Especially in the case of disaccharides, gas and acid may be produced from the products of heat action on the disaccharide, whether or not they could be produced from the disaccharide intentionally introduced. It is therefore especially necessary, if one is to use the simpler test, to know how and to what extent the heat of sterilization acts on the sugars in question.

That disaccharides, on heating, hydrolize into their component sugars is a well-known fact. C. S. Butler (1913) states,

"Some carbohydrates suffer inversion to a lower order if subjected to heat in the presence of weak acid or alkali, and in the finished product we find, for instance, that a supposed Shiga strain of the dysentery bacillus is producing acid in the maltose medium. As a matter of fact, it is simply showing its capacity to ferment glucose inverted from maltose."

It is the opinion among bacteriologists that maltose "breaks down" in this way even in the comparatively short period of sterilization. It was also thought that heating in the autoclave at fifteen pounds for fifteen minutes was more destructive to the sugars than heating in the Arnold for fifteen minutes on three successive days. So prevalent is this idea among bacteriologists that we now find in the text-books directions which say that "media which contain neither sugar nor gelatin . . . . may be sterilized in the autoclave at fifteen pounds for from fifteen minutes to one-half hour. Media which contain these must be sterilized by the fractional method" (Hiss and Zinsser, 1912). On the other hand, we are told that "in the breaking down of sugar media the time of sterilization has a greater effect than does the temperature" (Standard Methods of Water Analysis, 1912).

Since a great deal of dependence is placed on the fermentation of sugars, any knowledge concerning the chemical transformation of the sugars in the preparation of the media should be of value. It was the aim of this investigation: (a) to determine the behavior of sugars during sterilization; (b) to determine what form of sterilization is best suited to the sterilization of sugar media. Both chemical and bacteriological methods were employed to this end.

CHEMICAL METHODS

It was necessary to devise a method which would enable one to determine quantitatvely a disaccharide in the presence of a monosaccharide. The amount of monosaccharide would doubtless be very small, but it is a well-known fact that the presence of monosaccharides seriously interferes with the reactions of disaccharides. Attempts to remove one or more of the sugars by bacteria or yeasts, or to separate the sugars by means of the osazone reaction (Marquenne, 1891) met with no succcess.

Hinkel and Sherman (1907) have shown that a modification of the original Barfoed's solution can be used for the detection of monosaccharides. Their reagent is made as follows: 4.5 grams of copper acetate are dissolved in 100 cc. of water to

which have been added 2 cc. of a normal acetic acid solution. This solution must be stable enough to withstand heating for ten minutes on a boiling water bath. In the presence of monosaccharides the copper solution is reduced to cuprous oxide. This reddish precipitate is easily seen. Excessive boiling is apt to lead to hydrolysis of the disaccharide and care must be taken to avoid this. Each of the sugars to be studied gives, on hydrolysis, products which react with the above reagent.

Cohen used this method to distinguish between glucose and maltose in solutions containing less than 0.02 per cent of the former, and at least 0.2 per cent of the latter, "provided the tests are controlled by check experiments with known solutions of all the sugars." Roaf (1908) also studied the action of maltase on maltose by means of this solution, and was able to determine minute quantities of glucose in the presence of the maltose.

TECHNIQUE

Test tubes of large bore were used, since 25 to 30 cc. of liquid were to be tested. One per cent solutions of the various sugars in distilled water were prepared and sterilized in the autoclave for different periods of time, fifteen minutes, thirty minutes, one hour and two hours. Some of the solutions were also heated in the Arnold for fifteen, thirty, and forty-five minutes. Twenty cubic centimeters of the sugar solution to be tested were transferred to a test tube, and 5 cc. of the copper solution added. After a thorough shaking, the tubes were heated to 100°C. in a water bath for four minutes. A series of standards containing various amounts of the monosaccharides corresponding to the disaccharide used was treated in similar manner. If any reduction took place its extent could be estimated by comparing it with the standards. As the amount of reduction was sometimes very slight, the tubes were compared in a strong light against a black background.

The determinations were made on maltose, lactose, sucrose, and raffinose, since these are the polysaccharides most commonly used in bacteriological technique. All of these sugars were

Merck's Blue Label chemicals. The results are given in table 1. The figures represent the comparative degree of reduction.

From this table it will be seen that of the four sugars, raffinose is the most stable; then follow sucrose, lactose, and maltose in the order named. Sucrose shows hydrolysis only after prolonged heating, but both lactose and maltose show hydrolysis in every case, with one possible exception. The action on lactose in the Arnold becomes evident only after the second heating. It will also be noticed that according to these figures the hydrolysis in the Arnold is greater than in the autoclave. This is a point of practical importance, and is contrary to most of the views

TABLE 1

Comparative amounts of monosaccharides detected by Barfoed's method

TIME	LACTOSE	MALTOSE	SUCROSE	RAFFINOSE
Autoclaved 15 minutes	Trace	1	0	0
Autoclaved 30 minutes	1.5	3	0	0
Autoclaved 1 hour	2	Very great	Slight	0
Arnold first day	0	4	0	0
Arnold second day	1	Very great	0	0
Arnold third day	2	Very great	0	0

held in regard to the two methods. As has been previously pointed out, the autoclave has long been considered as having the greater hydrolytic effect on the sugars, yet the time factor in these experiments is evidently more important than the temperature factor. These results obtained from a study of pure sugars in water solution would not necessarily apply to culture media. The chemical methods used are not applicable to culture media; and the behavior of the sugars in culture media was therefore studied by bacteriological methods.

BACTERIOLOGICAL METHODS

Stock solutions of nutrient broth were prepared as follows:

Liebig's extract	0.3 per cent
Witte's peptone	1.0 per cent
Sugars	1.0 per cent
Reaction	Neutral to phenolphthalein

The sugars were added to the broth just previous to tubing. They were then sterilized as follows: fifteen minutes, thirty minutes, one hour, and two hours in the autoclave; and fifteen minutes on three successive days in streaming steam in the Arnold sterilizer.

TABLE 2

Acid produced by fermentation in sugar media after sterilization. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium*

Maltose inoculated with *B. dysenteriae*

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference*	Total acid	Difference
	cc.	*cc.*	*cc.*	*cc.*	*cc.*
Autoclaved 15 minutes	0.38	0.96	0.58	1.55	1.17
Autoclaved 30 minutes	0.45	1.23	0.78	1.24	0.79
Autoclaved 1 hour	0.73	1.73	1.00	1.62	0.89
Autoclaved 2 hours	0.92	1.74	0.82	1.95	1.03
Arnold 3 days	0.35	1.23	0.88	1.71	1.36
Lactose inoculated with B. paratyphosus					
Autoclaved 15 minutes	0.40	0.55	0.15	0.46	0.06
Autoclaved 30 minutes	0.54	0.78	0.24	0.60	0.06
Autoclaved 1 hour	0.68	0.97	0.29	0.73	0.05
Autoclaved 2 hours	0.87	1.30	0.43	1.17	0.30
Arnold 3 days	0.41	0.60	0.19	0.46	0.05
Sucrose inoculated with B. coli					
Autoclaved 15 minutes	0.35	0.50	0.15	0.41	0.06
Autoclaved 30 minutes	0.37	0.46	0.09	0.46	0.09
Autoclaved 1 hour	0.39	0.55	0.16	0.47	0.08
Autoclaved 2 hours	0.40	0.56	0.16	0.52	0.12
Arnold 3 days	0.39	0.46	0.07	0.41	0.02

Acid is produced by the Shiga strain of *B. dysenetriae* from glucose, but not from maltose. Inoculations with this strain were employed in the maltose experiments as a test for the production of glucose by hydrolysis. Similarly *B. paratyphosus* was used to detect hydrolysis of lactose, and *B. coli* was used for sucrose.

* Difference is found by subtracting acid of control tube from total acidity.

After incubation for twenty-four hours, 5 cc. of the culture were titrated hot against N/20 NaOH, using phenolphthalein as an indicator. The results given in the tables are the averages of ten titrations, expressed in cubic centimeters. Control titrations were made on the uninoculated broth, and this acidity was eventually subtracted from the acidity determined at the end of the twenty-four and forty-eight hour periods. The difference between these two figures was taken as the acidity caused by the fermentation of the monosaccharide. Broth with no added sugar was also inoculated, to determine what influence the muscle sugar might have.

TABLE 3

Acid produced in nutrient broth inoculated with same organisms used in Experiment 2. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium*

Nutrient broth inoculated with *B. dysenteriae*

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference	Total acid	Difference
	cc.	*cc.*	*cc.*	*cc.*	*cc.*
Autoclaved 15 minutes	0.25	0.30	0.05	0.32	0.07
Autoclaved 30 minutes	0.21	0.35	0.14	0.36	0.15
Autoclaved 1 hour	0.26	0.34	0.08	0.34	0.08
Autoclaved 2 hours	0.22	0.36	0.14	0.36	0.14
Arnold 3 days	0.27	0.37	0.10	0.34	0.07

Nutrient broth inoculated with *B. paratyphosus*

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference	Total acid	Difference
Autoclaved 15 minutes	0.25	0.42	0.17	0.37	0.12
Autoclaved 30 minutes	0.21	0.35	0.14	0.28	0.07
Autoclaved 1 hour	0.26	0.37	0.11	0.28	0.02
Autoclaved 2 hours	0.22	0.38	0.16	0.25	0.03
Arnold 3 days	0.27	0.36	0.09	0.27	0.00

Reference to the tables shows that sucrose gives no evidence of hydrolysis, whereas maltose and lactose show considerable hydrolysis, as evidenced by acid production. In the case of both of these sugars, more acid is produced in the tubes sterilized in the Arnold than in the tubes autoclaved for thirty minutes. Maltose seems to be broken down more than the lactose. This is in accord with the findings obtained by the chemical method applied to the pure sugar solutions.

An interesting fact was noticed in the case of lactose. The amount of acid found decreased during the second twenty-four hour period. The work of Kendall (1911) suggests the possibility that the organisms use up all the available sugar in the first twenty-four hour period, and next attack the protein,

TABLE 4

Acid produced by fermentation in sugar media after sterilization. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium*

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference	Total acid	Difference
	cc.	*cc.*	*cc.*	*cc.*	*cc.*
Maltose inoculated with *B. dysenteriae*					
Autoclaved 15 minutes	0.34	0.53	0.19	0.86	0.52
Autoclaved 30 minutes	0.44	0.76	0.32	0.96	0.52
Autoclaved 1 hour	0.53	1.30	0.77	1.35	0.82
Arnold 3 days	0.40	0.45	0.05	1.18	0.78
Lactose inoculated with *B. dysenteriae*					
Autoclaved 15 minutes	0.14	0.26	0.12	0.25	0.11
Autoclaved 30 minutes	0.20	0.31	0.11	0.20	0.00
Autoclaved 1 hour	0.37	0.72	0.35	0.29	0.08
Arnold 3 days	0.22	0.33	0.11	0.76	0.54
Sucrose inoculated with *B. dysenteriae*					
Autoclaved 15 minutes	0.17	0.22	0.05	0.20	0.03
Autoclaved 30 minutes	0.13	0.24	0.11	0.30	0.17
Autoclaved 1 hour	0.14	0.21	0.07	0.28	0.14
Arnold 3 days	0.12	0.20	0.08	0.33	0.21
Raffinose inoculated with *B. coli*					
Autoclaved 15 minutes	0.24	0.40	0.16	0.22	0.02
Autoclaved 30 minutes	0.26	0.40	0.14	0.35	0.09
Autoclaved 1 hour	0.29	0.36	0.07	0.36	0.07
Arnold 3 days	0.29	0.31	0.02	0.27	−0.02

setting free ammonia, which neutralizes the acid originally produced.

The amount of acid produced in maltose solutions is considerable, and it was thought that the stock sample might be somewhat hydrolyzed. A fresh supply was obtained from Merck and the tests were conducted as follows: Maltose, lactose, and

sucrose were inoculated with the "Y" Hiss strain of *B. dysenteriae;* the raffinose was inoculated with *B. coli.* The methods used were the same as those of the previous experiments. The results are found in tables 4 and 5, and it will be seen that they are similar to those of the first experiment.

The sterilization of sugar media by heating methods is evidently very destructive to the disaccharides, maltose and lactose. Media containing these sugars can be sterilized by filtering through unglazed porcelain. It might be expected that little or no chemical change would be produced by this treatment.

TABLE 5

Acid produced in nutrient broth inoculated with same organisms used in Experiment 4. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium.*

Nutrient broth inoculated with *B. dysenteriae*

	CONTROL	TWENTY-FOUR HOURS		FORTY-EIGHT HOURS	
		Total acid	Difference	Total acid	Difference
	cc.	*cc.*	*cc.*	*cc.*	*cc.*
Autoclaved 15 minutes	0.03	0.20	0.17	0.27	0.24
Autoclaved 30 minutes	0.10	0.18	0.08	0.29	0.19
Autoclaved 1 hour	0.11	0.24	0.13	0.28	0.17
Arnold 3 days	0.10	0.26	0.16	0.29	0.19

Nutrient broth inoculated with *B. coli*

Autoclaved 15 minutes	0.03	0.31	0.28	0.22	0.19
Autoclaved 30 minutes	0.10	0.33	0.23	0.21	0.11
Autoclaved 1 hour	0.11	0.35	0.24	0.23	0.12
Arnold 3 days	0.10	0.40	0.30	0.24	0.14

Accordingly, an experiment was conducted in which two lots of the same medium containing lactose were sterilized by two different methods. One part was heated for fifteen minutes at 15 pounds pressure in the autoclave. The other part was sterilized by filtering through a Chamberland filter. Every precaution was taken to keep the medium sterile. The tubes were placed in the incubator for twenty-four hours, and those showing contamination were discarded. These two lots of media were then inoculated with *B. paratyphosus B.*

TABLE 6

Comparison of autoclaving and filtering. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium*

	CONTROL TUBE	INOCULATED TUBE	DIFFERENCE
	cc.	*cc.*	*cc.*
Filtered broth	0.10	0.20	0.10
Heated broth	0.45	0.92	0.47

The filtered broth showed an acid production of 0.1 cc. N/20 which is about the limit of titration, while the broth heated in the autoclave titrated 0.4 cc. of alkali. It is evident that filtering the medium is a more sparing procedure.

THE INFLUENCE OF STERILIZATION ON THE REACTION OF SUGAR MEDIA

In the foregoing experiments the medium before sterilization was "neutral" (faintest tinge of pink to phenolphthalein) During sterilization the media which contained sugar became acid, the acidity increasing with the time. This had not been the case with peptone broth containing no sugar. The sugar solution in distilled water used for the Barfoed's test produced no acid during two hours sterilization in the autoclave. It thus appeared that both sugar and peptone, or other active substance in the peptone broth, are necessary for the development of acidity during sterilization.

A table (table 7) was compiled from data presented above. It will be noticed that peptone gives more acidity than the beef extract; the nutrient broth about the same as the peptone. However, when a sugar is added a great difference is noted. The addition of maltose or lactose gives rise to considerable acidity on sterilization. Sucrose shows some acidity and raffinose but little. It is interesting to note that the sugars which give the greatest acidity on sterilization are the ones which have been found above to break down most readily.

Hugo Schiff (1901–1902) showed that there is a reaction between formaldehyde and the amino group. In the amino acids, which

TABLE 7

Acid produced in sterilizing various media. Acidity expressed in cubic centimeters N/20 NaOH *in 5 cc. of medium*

	MEAT EXTRACT	PEPTONE	NUTRIENT BROTH	MALTOSE BROTH	LACTOSE BROTH	SUCROSE BROTH	RAFFINOSE BROTH
	cc.	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*	*cc.*
Autoclaved 15 minutes	0.13	0.24	0.25	0.38	0.40	0.35	0.25
Autoclaved 30 minutes	0.13	0.30	0.21	0.45	0.54	0.37	0.23
Autoclaved 1 hour	0.13	0.26	0.26	0.73	0.68	0.39	0.28
Autoclaved 2 hours	0.16	0.29	0.22	0.92	0.87	0.40	0.32
Arnold 3 days	0.13	0.28	0.27	0.35	0.41	0.39	0.32

contain the carboxyl group (COOH) and the amino group (NH_2) Schiff was able to neutralize the influence of the amino part on the carboxyl group by treating with neutral formaldehyde. The grouping

$$\begin{array}{ccl} & H & \\ & | & \\ - & C & - COOH \\ & | & \\ & NH_2 & \end{array}$$

is changed to

$$\begin{array}{ccl} & H & \\ & | & \\ - & C & - COOH \\ & | & \\ & N & \\ & | & \\ & CH_2 & \end{array}$$

Thus alanine, when treated with formaldehyde, is converted into methylene-alanine and water as follows:

$$\begin{array}{lclclclcl} & H & & H & & & H & & \\ & | & & | & & & | & & \\ CH_3 - & C & - COOH + & C & = O = & CH_3 - & C & - COOH + H_2O. \\ & | & & | & & & | & & \\ & NH_2 & & H & & & N - CH_2 & & \end{array}$$

Neutral solutions of amino acids are rendered acid on the addition of neutral formaldehyde. Since sugar may be considered as a polymer of formaldehyde and has similar reacting groups, it might easily be imagined that it would follow reactions similar to those mentioned above.

An experiment was devised to test this point. A 0.5 per cent solution of alanine was prepared, together with a 1 per cent solution of some old maltose, new maltose and raffinose. Solutions were then made which contained 0.5 per cent of alanine and 1 per cent of the sugars mentioned. Portions of each of these solutions were sterilized in the autoclave for periods of fifteen minutes, thirty minutes, and one hour. Five titrations were made on each solution, and the results are given in table 8. The titrations were made as in the previous experiments.

TABLE 8

The effect of alanine on the production of acid from sugars by autoclaving. Acidity expressed as cubic centimeters N/20 NaOH *per 5 cc. solution*

	LENGTH OF STERILIZATION		
	Fifteen minutes	Thirty minutes	One hour
	cc.	*cc.*	*cc.*
Alanine	0.00	0.00	0.00
Old Maltose	0.00	0.00	0.00
Alanine + Old Maltose	0.00	0.34	0.46
Fresh Maltose	0.00	0.00	0.00
Alanine + Fresh Maltose	0.00	0.10	0.30
Raffinose	0.00	0.00	0.00
Alanine + Raffinose	0.00	0.00	0.00

All of the solutions were neutral to phenolphthalein at the start of the experiment. The results show that the alanine alone, and the sugar alone, give no acidity. But in those solutions containing the mixture of alanine and maltose, acid is produced. The mixture of alanine and raffinose is neutral, even after sterilization for an hour. The production of acid is not the only index of chemical change. Those solutions which become acid also become colored. The depths of color, as well as the acid, increased as the length of sterilization was increased.

A similar experiment was performed, using maltose, lactose, and raffinose in solution with asparagin. The results were like those of the previous experiment.

TABLE 9

The effect of asparagine on the production of acid from sugars by autoclaving. Acidity expressed as cubic centimeters N/20 NaOH *per 5 cc. solution*

	LENGTH OF STERILIZATION		
	Fifteen minutes	Thirty minutes	One hour
	cc.	*cc.*	*cc.*
Asparagine	0.00	0.00	0.00
Maltose	0.00	0.00	0.00
Asparagine + Maltose	0.00	0.44	0.58
Lactose	0.00	0.00	0.00
Asparagine + Lactose	0.00	0.34	0.64
Raffinose	0.00	0.00	0.00
Asparagine + Raffinose	0.00	0.00	0.00

These experiments seem to bear out the fact previously indicated—that it is the presence of an unstable sugar molecule together with an amino acid which gives rise to the acidity of sterilization.

SUMMARY AND CONCLUSIONS

An attempt has been made to determine the extent of hydrolysis of certain disaccharides commonly used in bacteriological investigations, with the following results:

By means of Barfoed's method maltose and lactose were found to be hydrolyzed to a considerable extent in water solution. Sucrose and raffinose did not break down.

Heating in streaming steam for three successive days in the Arnold seems to hydrolyze lactose and maltose more than heating in the autoclave at 15 pounds for fifteen minutes.

By the use of bacterial cultures the hydrolysis of these sugars was studied in culture media, with similar results.

Peptone broths containing the sugars mentioned were subjected to sterilization in the Arnold in streaming steam and to various heatings in the autoclave at 15 pounds pressure. They

were then inoculated with strains of bacteria which would not ferment the sugar introduced.

Lactose and maltose broth, so inoculated, were found to be fermented by the organisms introduced. The amount of fermentation increased as the sterilization increased. It is assumed that this fermentation is due to the presence of monosaccharides in the broth.

By filtering sugar media this hydrolysis of the sugar can be avoided.

The sugars found above to be easily hydrolizable, namely, maltose, and lactose, formed acid when heated in culture media, or with alanine or asparagin in water solution; whereas raffinose, which is resistant to hydrolysis, formed no acid under like circumstances. The acidity produced in sterilization seems to be due to a reaction between the sugar and the amino groups.

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THE PRESENCE OF THE B. COLI AND B. WELCHII GROUPS IN THE INTESTINAL TRACT OF FISH (STENOMUS CHRYSOPS)

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The *B. coli* group, the *B. Welchii* group and other lactose-fermenting organisms have long been regarded as normal inhabitants of the intestinal tract of the homoiothermal or warm-blooded animals, and their presence outside of the body has been regarded as a very strong index of the presence of fecal pollution from those animals. The presence of these microorganisms in the intestinal tract of the poecilothermal or cold-blooded animals, however, has been a subject for dispute. A review of the literature does not seem to help the situation in the least, for we find the authorities evenly divided on the subject. The tendency in England and America seems to be to regard the *B. coli* group as the normal inhabitants of the higher animals only, while on the continent of Europe, especially in Germany, the interpretation has not been so strict. No opinions concerning the status of the *B. Welchii* group could be found, and such would be difficult to form because of the spore-forming abilities and anaerobic conditions necessary for the growth of that group of organisms.

All workers seem to agree, however, that the *B. coli* group is much more constant in the intestinal tract of the higher animals than of the cold-blooded animals. Hoag (1899) was able to isolate members of the *B. coli* group from the fish which he examined. Amyot (1902) examined the intestinal contents of 23 fishes, representing 14 species taken from Lake Erie, and was unable to isolate any lactose fermenters of the colon type.

Houston (1903) found members of the colon group in 13 per cent of the fishes examined, but concludes that the *B. coli* group is "either absent or present in small numbers in the intestines of fishes dredged from the sea in localities remote from sewage pollution." Whipple (1904) was unable to find the colon group in the intestinal tract of the trout, perch, and sunfish, taken from unpolluted waters, while Johnson (1904) was able to isolate these organisms 47 times out of the 67 fishes examined from polluted waters. Eyre (1904) isolated the members of the *B. coli* group from the intestinal tract of the sprat, dab, smelt, mullet, sole, skate and dogfish taken from traps two miles from the shore in unpolluted waters. Van Mallannah in an unpublished work reports positive results in 37.5 per cent of 32 fish examined, while Fromme states that 41 per cent of fishes examined responded positively to the *B. coli* tests. He concludes, however, that the *B. coli* group is only rarely present in the intestinal tract of the cold-blooded animals, while they are much more constant in the higher vertebrates. Bettencourt and Borges (1908) isolated 29 cultures of colon-like bacilli from the intestinal tract of 17 fishes, reptiles and amphibia, of which only two proved to be members of the colon group, although in these tests no enrichment medium was employed.

The purpose of this paper is to present some results as to the presence of the *B. coli* group and the *B. Welchii* group in the intestinal contents of fishes which were obtained at the Woods Hole Laboratory of the Bureau of Fisheries during the summer of 1916, while the writer was making an intensive study of the Bacteriology of the Food Fishes, especially the scup (*Stenomus Chrysops*).

The scup belong to the family Sparidae and to that large group of fishes known as "bottom feeders" such as the porgies, sea robins, drum fishes, tautog and sea bass which obtain their food supply from the floor of the ocean; hence, the character of the bottom on which the fish feed will influence the bacterial flora of their intestinal tract. Peck (1895) found that the food of the scup was somewhat varied but generally characteristic of the bottom fauna and flora. He found "at times very many

sand dollars (*Echinarachnius parma*) ground up with the sand and deep black mud of the bottom from which they were feeding, just above which the amphipods are usually abundant." Young specimens have often been found near the shores of coves and harbors feeding on young fishes.

The scup used in the following experiments were taken from the waters in and around the Woods Hole Laboratory of the Bureau of Fisheries. In some cases the fishes examined came from the government traps situated in Buzzards Bay in waters which were always coli-negative, and in other cases they came from the traps of the Marine Biological Laboratory situated either in Buzzards Bay or Martha's Vineyard Sound, both of which were free from any bacteriological evidence of fecal pollution, while other fish were caught by hook and line in Laky's Bay, an arm of Martha's Vineyard Sound, south of the Marine Biological Laboratory traps. The depth of the water from which the fishes were taken varied from a few feet near the shore in case of the traps to over 40 feet in Laky's Bay. In general the bottom seemed to be rocky and the waters were always free from the presence of lactose fermenters of intestinal origin (as shown by formation of gas in lactose broth).

The fishes were brought to the laboratory in clean baskets just as soon as possible after they were caught, and the following examination was made. After the scales were removed, the skin was thoroughly washed with 95 per cent alcohol and ignited. An incision was then made through the skin into the peritoneal cavity and the intestinal tract carefully dissected out, using instruments which were sterilized by constantly dipping in 95 per cent alcohol and lighting in the flame. As soon as the intestinal tract was dissected out, its exterior was seared with a hot scalpel at a distance of about two inches from the anus and then an incision was made with sterile scissors and the intestinal content allowed to drop into tubes of lactose broth, which were incubated at 37°C. It was impossible to graduate exactly the amount of fecal material which dropped into the tubes, but generally 5 to 10 grams of the intestinal tract content was used. Tubes which showed a posi-

TABLE

	LOT NUMBER												TOTAL
	1	2	3	4	5	6	7	8	9	10	11	12	
Source	B. T.	A.	S. T.	S. T.	B. T.	B. T.	B. T.	B. T.	B. T.	V. S.	V. S.	V. S.	
Date	7/20	7/25	7/27	8/2	8/5	8/7	8/21	8/22	8/23	8/24	8/29	8/30	
Number of fish examined	10	7	2	8	8	6	5	6	5	8	21	7	93
Gas in lactose broth:													
Positive	4	6	2	6	7	4	4	4	4	7	19	6	73(78.5%)
Negative	6	1	0	2	1	2	1	2	1	1	2	1	20(21.5%)
B. coli group isolated	4	6	1	2	4	0	2	2	2	4	7	3	37(39.8%)
Alone	4	5	1	2	4	0	2	2	2	1	1	3	27(29.0%)
With *B. Welchii*	*	1	0	0	0	0	0	0	0	3	6	0	10(10.8%)
B. Welchii group isolated	*	1	0	1	2	1	0	1	0	4	16	2	28(30.1%)
Alone	*	0	0	1	2	1	0	1	0	1	10	2	18(19.3%)
With *B. coli*	*	1	0	0	0	0	0	0	0	3	6	0	10(10.8%)
Condition of water	0	+	0	0	0	0	0	0	0	0	0	0	
Total positive fish (Welchii or Coli)	4	6	1	3	6	1	2	3	2	5	17	5	55(59.2%)
Total negative fish	6	1	1	5	2	5	3	3	3	3	4	2	38(40.8%)

* No examination.

S. T. = Sound trap; A. = Aquarium; B. T. = Bay trap; V. S. = Vineyard Sound; + = *B. coli* present.

tive gas production by the forty-eighth hour were plated out on litmus lactose agar, and transplants were identified as members of the *B. coli* group. A Gram-negative, rounded and aerobic bacillus, producing gas and acid in lactose broth, and no liquefaction of gelatine, was taken as the criterion of membership in the *B. coli* group. All tubes which showed gas were examined for the *B. Welchii* group by the following procedure. One cubic centimeter of the lactose broth tube showing gas production was inoculated into a tube of litmus milk which was then sealed with paraffin. These tubes were heated at a temperature of 80°C. for ten minutes after which they were incubated at 37°C. for forty-eight hours. Vigorous gas production, with the subsequent expulsion of the paraffin plug, acid reaction, "stormy fermentation" and odor of butyric acid were regarded as indicative of the presence of the *B. Welchii* group.

The results obtained by the above bacteriological examination of the intestinal content of the scup for the presence of the *B. coli* group and the *B. Welchii* group may be summarized in the accompanying table.

CONCLUSIONS

The intestinal content of 93 scup (*Stenomus chrysops*) taken from unpolluted waters in and about the Woods Hole Laboratory of the Bureau of Fisheries was examined bacteriologically for the presence of the *B. coli* and the *B. Welchii* group with the following results:

1. The intestinal content of 73 (78.5 per cent) scup examined contained lactose-fermenting organisms.

2. Members of the *B. coli* group were isolated from the intestinal content of 37 (39.8 per cent) of the scup examined, in 27 (29.0 per cent) cases alone and in 10 (10.8 per cent) cases in company with members of the *B. Welchii* group.

3. The members of the *B. Welchii* group were isolated from the intestinal content of 28 (30.1 per cent) of the scup examined, in 18 (19.3 per cent) cases alone and in 10 (10.8 per cent) cases in company with the members of the *B. coli* group.

4. The intestinal content of 55 (59.2 per cent) of the scup examined contained organisms of either the colon or the Welchii type.

5. The presence of bacteria in the intestinal tract of the scup seems to be correlated with the amount and type of food present.

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NITROGEN-ASSIMILATING ORGANISMS IN MANURE[1]

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In a recent article from the Wisconsin Agricultural Experiment Station, Tottingham[2] called attention to a gain in nitrogen in fermenting manures. Because of the practical importance of stable manure for agriculture, it was thought advisable to study the nature of the organisms concerned with the fixation of nitrogen in manures.

EXPERIMENTS AND RESULTS

During the winter of 1916 experiments were planned to determine the occurrence and nitrogen-assimilating power of the organisms in manure. Samples of manure were prepared as follows: A mixture of manure consisting of two parts of fresh horse manure and one part of fresh cow manure was added to finely chopped wheat straw. One-half kilo of straw was taken to every 6.5 kilos of the horse-cow manure mixture.

The presence of nitrogen-fixing organisms was determined by the gain in nitrogen in liquid and solid cultures. One hundred cubic centimeter portions of mannitol solution, in one liter Erlenmeyer flasks, were inoculated with 10 grams each of the manure mixture. Immediately after inoculation one-half of the cultures were treated with strong sulphuric acid or sterilized in the autoclave. These served as controls. The remaining cultures were incubated for varying intervals of time. At the end of the

[1] Published by permission of the Director of the Wisconsin Agricultural Experiment Station.

[2] TOTTINGHAM, W. E. 1916 Jour. Biol. Chem., v, 24, no. 3, p. 221.

incubation period determinations of total nitrogen content were made on the controls, as well as on the cultures. The difference in the amount of nitrogen shows the increase due to biological factors.

EFFECT OF AGE OF MANURE ON NITROGEN FIXATION

The manure mixture was prepared as previously described, stored at room temperature, and at intervals of two and eight weeks composite samples were weighed into the mannitol solution. In each test 10 flasks were used, 5 controls and 5 cultures. The controls received 25 cc. of concentrated sulphuric acid immediately after inoculation. The cultures were kept at 28°C. At the end of three weeks, total nitrogen analyses were made of the entire contents of the flask. Table 1 shows the milligrams of nitrogen in each culture inoculated with manure two and eight weeks old. In mannitol solution manure two weeks old gave an increase of 6 mgm.; eight weeks old an increase of 6.4 mgm. of nitrogen. The age of the manure, at least within a certain range, apparently has very little effect on the gain in nitrogen. In the manure itself Tottingham found that after

TABLE 1

Effect of age of manure on gain in nitrogen

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION					
		Two weeks old			Eight weeks old		
			Average	Gain		Average	Gain
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	Control	37.5			68.7		
2	Control	37.9			68.0		
3	Control	37.1	37.4		64.7	66.8	
4	Control	37.2			65.9		
5	Control	37.1					
6	Bacteria	43.1			73.1		
7	Bacteria	43.6			73.0		
8	Bacteria	43.6	43.4	6.0	73.1	73.2	6.4
9	Bacteria	43.3			73.8		
10	Bacteria	44.2					

two to four weeks of fermentation there was a gain in the absolute amount of nitrogen; after twelve weeks, a loss. The increase in nitrogen due to the organisms of fermenting manure compares favorably with that obtained with the organisms of field soil. For example, 10 grams of soil in 100 cc. of mannitol solution gave an increase in nitrogen of 6 to 10 mgm.[3] The apparent increase in the nitrogen of the older sample of manure is no doubt due to a difference in the moisture content. At the time of inoculation the fresh manure mixture contained much more water than the old mixture.

EFFECT OF TEMPERATURE OF INCUBATION ON GAIN IN NITROGEN

In order to determine the temperature suitable for the growth of the nitrogen-assimilating organism or organisms of manure, when inoculated in mannitol solution, duplicate sets of cultures were prepared. One set was kept at 28°C. and the other was incubated at 37°C. The manure inoculum was eight weeks old. Nitrogen analyses after twenty-one days gave the results indicated in table 2. The data show that the organisms of manure fix more nitrogen at 28°C. than at 37°C.

TABLE 2

Effect of different temperatures of incubation on gain in nitrogen

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION					
		Incubated at 28°C.			Incubated at 37°C.		
			Average	Gain		Average	Gain
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	Control	41.9			41.9		
2	Control	42.0	41.9		42.0	41.9	
3	Control	41.7			41.7		
4	Bacteria	51.4			45.6		
5	Bacteria	48.1	49.7	7.8	47.5	46.5	4.6
6	Bacteria	49.7			46.5		

[3] Unpublished results of this station.

EFFECT OF HEAT ON THE NITROGEN-ASSIMILATING ORGANISMS OF MANURE

It was next arranged to study the relation of the nitrogen-fixing organisms of manure to partial sterilization. Six flasks of mannitol solution were inoculated with the manure mixture and then heated to 75°C. for ten minutes. If the gain in nitrogen is due to a spore-bearing organism, it is probable that this treatment will not have any decided effect on the amount of nitrogen assimilated. The data of table 3 furnish an answer to the question. The total gain after eighteen days' incubation at 28°C. amounted to 2.2 mgm. or about one-third the amount fixed in the unheated flasks. Apparently fermenting manure contains a nitrogen-fixing organism which is resistant to heat—a spore-forming organism. However, the total gain in nitrogen from the resistant organism, or organisms, is far less than that from the natural flora. The evidence indicates the presence of several kinds of nitrogen-fixing organisms.

TABLE 3

Effect of heating manure on the nitrogen-fixing flora

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION		
		Heated to 75°C.		
			Average	Gain
		mgm.	*mgm.*	*mgm.*
1	Control	41.9		
2	Control	42.0	41.9	
3	Control	41.7		
4	Bacteria	43.8		
5	Bacteria	43.9	44.1	2.2
6	Bacteria	44.5		

EFFECT OF TIME OF INCUBATION ON NITROGEN FIXATION

Microscopic examinations of the mannitol solution inoculated with manure showed a profuse growth of microorganisms, largely bacilli. About fifteen days after inoculation the bacterial film became covered with a mold growth. An attempt was

made to measure the relation of the molds to the increase in nitrogen. Advantage was taken of the slow development of the molds in comparison with the bacteria. Cultures were prepared and incubated for nine and twenty-one days.

Nitrogen assimilation takes place independently of mold growth, as shown in the figures of table 4. The total number of milligrams fixed was almost as great after nine days as after fifteen days.

Attention was next directed to the gain in nitrogen with pure cultures isolated from the manure mixture.

TABLE 4

Effect of time of incubation on the nitrogen-fixing flora

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION					
		Incubated for nine days			Incubated for twenty-one days		
			Average	Gain		Average	Gain
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	Control	35.3			37.5		
2	Control	36.7			37.9		
3	Control	36.6	36.3		37.1	37.4	
4	Control	36.1			37.2		
5	Control	36.6			37.1		
6	Bacteria	40.6			43.1		
7	Bacteria	41.0			43.6		
8	Bacteria	42.3	41.5	5.2	43.6	43.4	6.0
9	Bacteria	42.2			43.3		
10	Bacteria	41.3			44.2		

NITROGEN FIXATION WITH PURE CULTURES OF BACTERIA ISOLATED FROM MANURE

Here again, inoculations from the manure were made into mannitol solution. The cultures were examined microscopically at various intervals. During the early stages of growth gas bubbles appeared on the top of the liquid, and later there developed a tough, gelatinous film on the surface. At first the film consisted solely of bacteria, later of bacteria and molds. Microscopic mounts made from the film and treated with Gram's

iodine solution showed the presence of organisms which stained a faint golden color; but Azotobacter organisms were never found.

Dilutions were made using mannitol agar and mannitol-manure-extract agar. The last medium was made by replacing the distilled water with a water extract from the manure. A series of plates was poured. From these plates isolated colonies were transferred to agar slopes. In every case there developed a more abundant growth on the mannitol-manure agar than on the mannitol agar alone. As soon as the agar slopes showed abundant growth, a loopful of the culture was shaken vigorously with sterile water and sand and then replated.

Because of their frequent occurrence in plate cultures, four organisms were selected for the first test. These were designated as numbers 1, 2, 3, and 4.

The nitrogen-assimilating power of cultures numbers 1, 2, 3, and 4 was tested on mannitol films. For this purpose 100 cc. portions of mannitol agar in one liter Erlenmeyer flasks were used. The flasks were inoculated with a 1 cc. suspension of the various organisms. The cultures were incubated at 28°C. and at varying intervals enough sterile water added to keep the surface moist. Nitrogen assimilation was studied on mannitol-manure-extract agar in addition to mannitol agar. Microscopic examination showed moderate growth on the mannitol agar and profuse growth on the mannitol-manure extract agar. After twenty-one days of incubation the cultures were analyzed for total nitrogen. All of the organisms assimilated a small amount of nitrogen. On mannitol agar the gain amounted to about 1.5 mgm.; on mannitol-manure extract agar about 2 mgm.

In view of the slight gain in nitrogen of cultures 1, 2, 3, and 4, it was decided to attempt to isolate other forms from the manure mixture.

Three organisms, 5, 6, and 7 were isolated and their nitrogen-assimilating power studied. The average of five parallel analyses showed that culture 5 fixed 4.7 mgm. of nitrogen on mannitol-manure-extract agar and only 1.7 mgm. on mannitol agar.

TABLE 5

Nitrogen assimilation by culture 5 on mannitol-manure-extract agar

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF AGAR		
		Culture 5		Gain
			Average	
		mgm.	*mgm.*	*mgm.*
1	Control	16.5		
2	Control	16.5		
3	Control	16.3	16.5	
4	Control	16.3		
5	Control	16.9		
6	Bacteria	21.5		
7	Bacteria	21.0		
8	Bacteria	21.3	21.2	4.7
9	Bacteria	21.2		
10	Bacteria	21.0		

A repetition of this test on agar films and liquid cultures of mannitol-manure-extract gave similar results. Organism 5 when grown in mannitol-manure-extract medium, liquid or solid, assimilated about 4.5 mgm. of nitrogen. Although the gain in nitrogen is not as great, this organism compares very favorably with Azotobacter. It is no doubt one of the chief factors in the assimilation of nitrogen in manure.

The same procedure was followed in studying nitrogen fixation with cultures 6 and 7. Culture 7 failed to show any in-

TABLE 6

Nitrogen assimilation by culture 6 on agar and in solution

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION					
		Mannitol-manure extract agar			Mannitol-manure extract solution		
			Average	Gain		Average	Gain
		mgm.	*mgm.*	*mgm.*	*mgm.*	*mgm.*	*mgm.*
1	Control	13.4	13.3		13.5	13.5	
2	Control	13.2			13.5		
3	Bacteria	18.5	19.0	5.7	16.7	16.6	3.1
4	Bacteria	19.5			16.5		

crease in nitrogen. The increase in nitrogen due to the growth of culture 6 is clearly seen from the figures in table 6. When grown on agar films the organism fixed more nitrogen than in solution.

A review of the data obtained with pure cultures shows that the gain in nitrogen is not as great as that obtained with manure itself. Apparently the presence of solid manure in the solution is not essential to nitrogen fixation. This fact is brought out very clearly by results presented in table 7. Here a water suspension of the manure was used to inoculate sterile mannitol-manure-extract. After sixteen days the increase in nitrogen amounted to 8.2 mgm.

TABLE 7

Nitrogen assimilation in mannitol-manure extract solution inoculated with manure

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF SOLUTION		
			Average	Gain
		mgm.	*mgm.*	*mgm.*
1	Control	9.5		
2	Control	9.5		
3	Control	9.4	9.3	
4	Control	9.1		
5	Control	8.9		
6	Bacteria	17.4		
7	Bacteria	17.6		
8	Bacteria		17.5	8.2
9	Bacteria	17.3		
10	Bacteria	17.7		

COMPARISON OF THE AMOUNT OF NITROGEN ASSIMILATED BY AZOTOBACTER AND BY ORGANISMS FROM MANURE

In this test a pure culture of Azotobacter isolated from garden soil was used to inoculate flasks of mannitol-manure agar. The same plan was followed as in previous experiments. Three weeks after inoculation, the Azotobacter cultures were analyzed. Table 8 gives the results of the total nitrogen determinations. Azotobacter fixed 8 mgm. of nitrogen. A comparison of the gain in nitrogen with manure bacteria and with Azotobacter fails to show any marked difference.

TABLE 8

Nitrogen assimilation by Azotobacter in mannitol-manure extract agar

NUMBER	TREATMENT	NITROGEN IN 100 CC. OF AGAR		
		Azotobacter culture		Gain
			Average	
		mgm.	*mgm.*	*mgm.*
1	Control	12.6		
2	Control	12.7		
3	Control	12.5	12.6	
4	Control	12.6		
5	Control	12.6		
6	Bacteria	20.3		
7	Bacteria	20.6		
8	Bacteria	20.3	20.6	8.0
9	Bacteria	20.7		
10	Bacteria	20.9		

NITROGEN ASSIMILATION BY A MIXTURE OF THE PURE CULTURES ISOLATED FROM MANURE

Since the tests with pure cultures of organisms from manure failed to show a large gain in nitrogen, another experiment was carried out to study the nitrogen-fixing power of various combinations of organisms 5, 6, and 7. The results of this study are shown in table 9.

TABLE 9

Nitrogen assimilation by a mixture of pure cultures

TREATMENT	NITROGEN IN 100 CC. OF SOLUTION						
						Average	Gain
	mgm.					*mgm.*	*mgm.*
Control	9.5	9.5	9.4	9.1	8.9	9.3	
Culture 5	12.3	12.5	12.3	12.6	12.5	12.4	3.1
Culture 6	11.7	11.6	11.9	11.5	11.4	11.6	2.3
Culture 7	9.8	9.4	9.6	9.3	8.9	9.4	0.1
Cultures 5 and 6	12.4	12.3	12.8	13.1		12.7	3.4
Cultures 5 and 7	12.3	11.9	12.5	12.0	12.4	12.2	2.9
Cultures 6 and 7	12.0	12.0	11.8	11.7	12.0	11.9	2.6
Cultures 5, 6, and 7	11.8	11.6	11.8	11.7	11.8	11.7	2.4

From the data it is very evident that organism 5 in pure culture fixed as much or more nitrogen than in mixed cultures containing organisms 6 and 7. Apparently there are present in fermenting straw manure several nitrogen-fixing organisms. Among these 5 plays an important part.

CLASSIFICATION

Since culture 7 failed to show any decided gain in nitrogen, it was not included in this study. Cultures 5 and 6 apparently belong to the same group. Therefore, data are presented for only one culture.

From a review of the literature, it appears that the organism responsible for a large part of the gain in nitrogen in manure is an undescribed form. The specific name, *azophile,* is suggested. In relation to nitrogen fixation *B. azophile* is of especial interest. In fermenting manure or in culture media this organism will assimilate large amounts of atmospheric nitrogen. The total gain in nitrogen with pure cultures of *B. azophile* is almost as large as that obtained with Azotobacter.

Although data concerning the general occurrence of this organism are not ready for publication, it seems very probable that *B. azophile* is widely distributed in nature. The subject of nitrogen-fixing bacteria of manure, is receiving further study.

MORPHOLOGY AND PHYSIOLOGY OF BACTERIUM AZOPHILE, N. SP.

Morphological characters

The organisms are rods, occur singly with rounded ends, 0.7 to 0.8 micron wide by 1.4 to 1.8 microns long. Capsules are formed when the organism is grown on mannitol-manure-extract agar. No spores have been observed. It is motile in the hanging drop, is Gram-positive, and stains easily with ordinary aniline dyes.

Cultural characters

Agar plates. On plain agar at 28°C. surface colonies are less than 1 mm. in diameter in twenty-four hours. After three days the diameter increases, in some cases to 3 mm. Colonies are round, smooth, convex, and glistening. On mannitol-manure-extract agar growth develops much faster and is more profuse. In five days the growth with the extract agar is very profuse. When a loop is inserted growth does not appear viscous but can be picked up in cakes. On mannitol agar the growth is slower than on plain agar, finally attaining a rather heavy growth after five to six days.

Gelatin stab. Gelatin is liquefied completely, the liquefaction being of medium rapidity. Growth downward is very slow. It develops on surface.

Agar stabs. Growth is 2 mm. or more at surface in twenty-four hours. It remains at surface, is smooth and shining.

Agar slants. On bouillon agar a light orange pigment develops. Growth is abundant and wrinkled. On mannitol agar and mannitol-manure-extract agar growth is smooth. With the latter medium the growth is very profuse.

Mannitol-manure-extract solution. In mannitol-manure-extract solution, after twenty-eight days, growth appears very heavy and liquid begins to "lopper," provided the solution is maintained in shallow layers.

Beef bouillon. A heavy membranous growth is developed which does not fall to the bottom readily.

Potato slopes. Growth is moderate and does not spread over entire surface. It is elevated, shining, and of a brownish color.

The slopes are not softened in ten days. A diastatic action is shown when tested with Fehling's solution.

Milk. Milk begins to digest in four to six days. A gelatinous pellicle is formed near the surface. Digestion continues downward slowly. Milk does not seem to curdle. No acid is produced in litmus milk.

Fermentation tubes. No gas is formed with sucrose, glucose, or lactose in bouillon. Growth takes place in all three media, but only on the surface. No acid is produced.

Nitrate broth. Nitrates are reduced to nitrites and finally to gaseous nitrogen or oxides of nitrogen.

Peptone solution. Heavy growth is developed near surface which later falls to bottom. Indol is produced.

Glycerol solution. Heavy membranous growth results. No acid is found when tested with blue litmus paper.

Aerobism. The organism is apparently a strict aerobe. It grows only in the open end of fermentation tubes, with ordinary sugars present; in agar stab, growth is on the surface only, and not at junction made by pouring melted agar on a column of solid agar. This organism's group number is 211.3331[4]23 according to the chart of the Society of American Bacteriologists.

SUMMARY

1. Nitrogen assimilation in fermenting manures is due to the activities of certain forms of bacteria.

2. Apparently there are several groups of bacteria concerned with nitrogen assimilation in manure.

3. *B. azophile*, n. sp., which occurs abundantly in fermenting manures, seems to be the chief organism responsible for the increase of nitrogen.

4. The increase in nitrogen, when the organism is grown in a manure extract medium, amounts to from 3 to 5 mgm. per 100 cc. of solution.

5. 28°C. seems to be a favorable temperature for the growth of the nitrogen-assimilating organisms of manure.

[4] Non-chromogenic on some media; chromogenic on bouillon agar and on potato.

NEW DIFFERENTIAL PLATING METHODS FOR B. BIFIDUS (TISSIER) AND B. ACIDOPHILUS (MORO)

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In connection with the study of fecal bacteria the following media and methods have been found useful for the isolation of aciduric forms, especially for quantitative determinations of *B. bifidus* and *B. acidophilus*.

B. BIFIDUS

With laboratory media in common use the isolation of *B. bifidus* from fecal material containing large numbers of *B. coli* is not easy. The preparation of anaerobic plates is necessary, and with glucose agar, which is ordinarily employed, the few colonies of *B. bifidus* developing are generally overshadowed by those of *B. coli*. There is also no well marked difference between the types of colony formation, both surface and deep, of these bacteria in ordinary glucose agar. I have found during the course of a study of the Gram-positive aciduric bacilli of the intestinal tract that solid media prepared with an infusion of beef liver, instead of muscle tissue as a base, is particularly favorable. For the isolation of *B. bifidus* a glucose blood liver agar is employed. In brief, the preparation of this medium is as follows: Cut 500 grams of beef liver in small pieces and add to 1000 cc. of distilled water, boil for two hours in a double boiler, filter through flannel and cotton, and to the filtrate add 10 grams of peptone and 20 grams of agar. Heat in flask in the Arnold for one hour, adjust to the reaction desired and clear with eggs if necessary; to the clear filtrate add 10 grams of glucose and 1 gram of di-potassium phosphate. For *B. bifidus* the medium is

titrated to + 1 to phenolphthalein, and to each 10 cc. about 1 cc. of sterile defibrinated rabbit blood is added, just before the plate is poured. For its optimum development *B. bifidus* requires a certain degree of anaerobiosis, but as will be shown below, it is not an obligate anaerobe, and will grow fairly well aerobically on this medium.

To obtain the reduction of oxygen favorable for the luxuriant development of *B. bifidus*, it has been found advantageous to utilize Nowak's (1908) suggestion for the partial exhaustion of oxygen through the action of a member of the subtiloid group of bacteria. As far as I am aware this method has not been applied hitherto to plate cultures. Perhaps the nearest approach is Horton's (1914) divided test tube device. A plate, however, offers obvious advantages over a tube in that a larger surface of medium is available for seeding and the growth may be readily inspected. The apparatus used by the writer is similar to the anaerobic plate described by Zinsser (1906), except that the lower larger dish contains agar seeded with *B. cereus* (*B. subtilis* would probably prove satisfactory) instead of the pyrogallic acid sodium hydrate mixture.

A Petri dish 10 cm. in diameter and at least 2 cm. high is selected and the glucose blood liver agar is poured into it, taking care that the sides of the dish are kept free from the medium. The dish should be placed in the incubator until the sides are dry and there is no obvious moisture on the surface of the medium, when it may be seeded. This drying is important, as moisture on the sides of the dish may permit contamination by the culture used to absorb the oxygen. Into the cover of a Petri dish about 12 cm. in diameter are next poured about 15 cc. of nutrient 3 per cent agar seeded with the *B. cereus* culture. Before this agar has quite solidified the half-dish containing the seeded liver blood agar medium is inverted and placed in it. The agar in the lower plate on solidifying forms a seal. The apparatus is placed in the incubator in a moist chamber to prevent the exposed agar medium from drying out.

The *B. bifidus* colonies are visible in twenty-four hours, but are not especially distinctive in appearance. After forty-eight

hours incubation, however, they may be readily recognized as raised, more or less *globular*, opaque colonies 1 to 3 mm. in diameter, *buff to reddish brown in color.* In fact the principal novelty in this mode of cultivation lies in the distinctive appearance of the *B. bifidus* colonies (fig. 2), while the method used for obtaining conditions of partial anaerobiosis is of secondary importance. Most strains of *B. acidophilus* form on this medium flat, dingy colonies with a serrated edge, although a few produce more or less convex whitish or yellowish growths. The *B. coli* colonies are easily distinguished. Hence, if the quantitative count of the viable *B. acidophilus* or *B. coli* has been determined for the specimen, by ascertaining the ratio of the colonies of *B. bifidus* appearing on the plate to those of *B. acidophilus* or *B. coli*, the count for *B. bifidus* may be estimated.

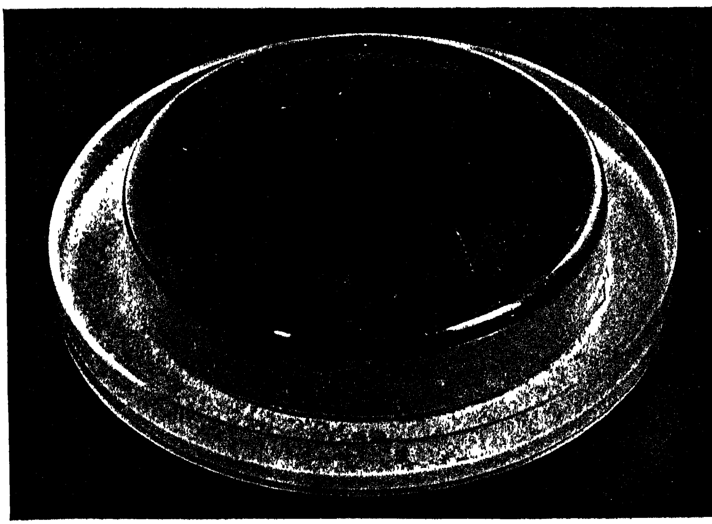

FIG. I. ANAEROBIC PLATE FOR B. BIFIDUS

Glucose blood liver agar is so favorable for *B. bifidus* that it will grow on this medium even under aerobic conditions. The colonies in primary plate cultures, however, are generally very small, and appear after forty-eight hours incubation as minute colorless cones. Some strains adapt themselves quickly to

aerobic conditions and give rise to a rather thick staphylococcus-like growth, while others continue to produce a very thin growth on slants of this medium. Both types may be kept alive indefinitely growing on slants under aerobic conditions, if transplants are made about every seven days.

With most strains of *B. bifidus* aerobic growth on liver glucose agar tends to encourage polymorphism, the profuse and variegated branching giving rise at times to coral-like aggregations.

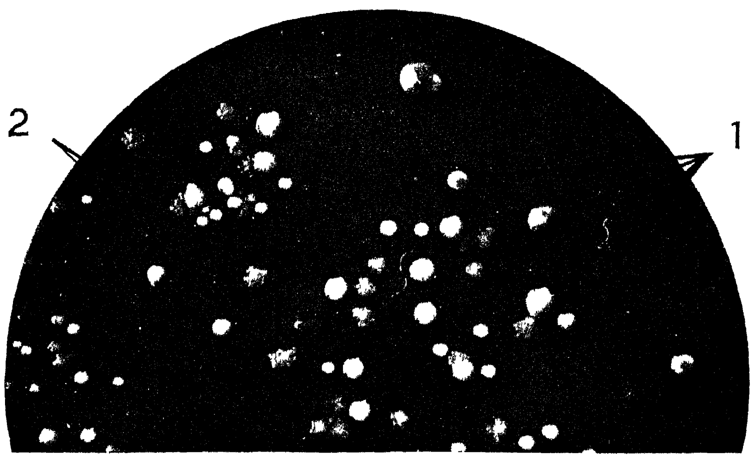

FIG. II. PHOTOGRAPH, SLIGHTLY ENLARGED, OF COLONIES ON A GLUCOSE BLOOD LIVER AGAR PLATE (ANAEROBIC) SEEDED FROM A DOG'S STOOL. 1. B. BIFIDUS COLONIES. 2. B. ACIDOPHILUS COLONIES.

Also, bifid forms are more likely to develop on liver medium than on media prepared with muscle tissue infusion as a base, when cultivated under conditions of partial anaerobiosis.

B. ACIDOPHILUS

Acid glucose agar or oleate glucose agar has generally been recommended heretofore for the cultivation of *B. acidophilus.* I have not found either of these media satisfactory for a quantitative determination of bacilli of this type in fecal specimens. In searching for a substitute it was found that glucose liver agar constitutes a decidedly favorable medium for bacteria of this

type, and furthermore that the typical colony formation facilitates quantitative determinations. The medium is prepared as described above for *B. bifidus*, except that no blood is added to it. *B. acidophilus* will develop on this medium with a range of reaction from neutral to phenolphthalein to +5 acid. A reaction of +3 acid appears to be most favorable, but for fecal work +4 acid is used in order to inhibit the development of streptococci and most strains of *B. coli*.

With a little experience the colonies formed by members of the *B. acidophilus* group may be recognized readily. The type most frequently encountered forms a small fluffy deep colony resembling a fleck of cotton. Certain other strains form globular deep colonies with a serrated border. Acid-tolerating colon bacilli give rise to lenticular deep colonies or sharp pointed triangular ones. Anaerobic plates are not necessary, as with this medium the development of the *B. acidophilus* colony is quite as satisfactory under aerobic as under anaerobic conditions.

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TRICHOMONASIS OF CHICKS: A NEW AND HIGHLY FATAL DISEASE

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During the spring of 1916 a highly fatal disease of chicks was discovered on a ranch near Puyallup, Washington, by Mr. F. W. Breed, poultry expert for the Fischer Flouring Mills Company of Seattle. Nine hundred chicks were hatched at one time from a thrifty stock of white leghorns. Ten days after hatching they developed a disease which rapidly reduced their number to less than one hundred. Sick specimens were brought to the laboratory for the determination of the cause of the trouble. Samples of the feed employed were also furnished to determine whether it might be the source of the disease. Later the same malady was discovered in the large poultry plant of Firland Sanitarium, Seattle, and also at other ranches in the Puget Sound region.

Autopsies revealed no marked lesions and afforded no clue as to the cause. It was natural to assume that the trouble was intestinal in origin, and that it might be either (a) bacterial, (b) protozoal, or (c) fungal. All of these alternatives were tested out on numerous specimens furnished to the laboratory. Bacterial cultures from the organs and heart's blood remained negative when made from killed sick chicks. From specimens which had died *Bact. coli* was isolated; but it never caused disease when fed to sound young chicks six days old.

The intestinal contents were examined for molds, as it appeared quite probable that moldy feed might be the source of the trouble. At the same time culture plates were made from the intestinal contents on agar especially suited to the culture of molds (agar, 20 grams; sucrose, 10 grams; potato water, 200 cc.;

water, 800 cc.). Only three species of molds were found and these were always few in number. In some of the chicks no molds were found. Feeding experiments with the molds isolated (which included *Aspergillus fumigatus*) proved negative, though the faeces showed innumerable colonies of the molds fed. The feed supplied showed few molds and these were of the same species as occurred in the chicks. Further, the feed was given to young control chicks which always throve on it in our laboratory. It appeared certain, therefore, that molds could not be the cause of the difficulty.

Examination of the intestinal contents was also made for coccidia and other protozoa, but they were apparently absent. Blood slides were always negative. For the time being the discovery of the cause of the disease seemed hopeless. At this stage of the investigation it was noticed in one of the autopsies that a caecum was slightly enlarged. The contents of this caecum were examined and revealed the presence of numerous flagellate protozoa. Subsequent examination showed that a single species was present, frequently in overwhelming numbers; that it was not present in healthy stock, or in newly hatched chicks; that healthy chicks kept in cages with sick chicks developed the disease and showed the protozoan in the caęca; and finally that control chicks did not develop the disease when kept under identical conditions. We concluded, therefore, that this protozoan is the cause of the disease.

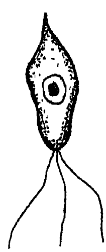
DESCRIPTION OF CHICK DISEASE

The disease apparently attacks young chicks only, for stock that is a month old proves resistant, and chronic cases that reach this age usually recover. The disease appears the second week after hatching, most commonly about the tenth day. Diarrhoea is absent in the early cases, but is present in the chronic stage. In the acute stage the chick may succumb within a day or two, and only the more vigorous birds enter the chronic stage. The chicks droop, huddle together, but do not refuse to eat. The wings drag, eyes are kept closed, and

the heads are pale. The temperature is subnormal and is commonly about 102°. On autopsy no lesions are found save a generally anaemic condition. The caeca may be slightly enlarged and the contents are thin and slimy in proportion to the severity of the disease. The crypts in the mucosa are invaded by the protozoa which form nests in them, frequently in pure culture. If the chicks develop diarrhoea, the protozoan may be found in the intestine and in the voided mucus. Commonly the chick dies in two or three days. Apparently the disease does not appear until late spring and summer, the earlier hatches during the cooler weather escaping it.

DESCRIPTION OF THE ORGANISM

The protozoan is a flagellate belonging to the genus *Trichomonas*. It presents two forms, one pear-shaped, the other globular. The pear-shaped form has a distinct tail, and three flagella situated at the head end, which are nearly the length



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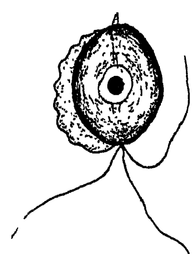

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FIG. 1. TRICHOMONAS PULLORUM. × 2000

The pear form or active stage in which the animal moves about by means of flagella, and also shows amoeboid movement.

FIG. 2. TRICHOMONAS PULLORUM. × 2000

The globular form in which the undulating membranes are kept in constant motion.

of the body. The globular form looks like another organism, but the change from one form to the other has repeatedly been observed. In this stage it displays on one side an undulating

membrane which is kept in constant motion; a spine-like process or axostyle is seen at the posterior end. The pear form is the active stage when the organism swims about by means of its flagella and also exhibits distinctly amoeboid movements. This is the only form found in the caecal crypts, but in the lumen it readily assumes the globular type. The protoplasm is finely granular, and the nucleus is situated centrally. The size varies considerably, ranging from 3.5 by 7 μ to 5.5 by 9 μ; the globular form is shorter and broader. The accompanying figures will supplement the description and perhaps clarify it. Since the organism is apparently different from the species described in the available literature (Doflein, 1909; Kolle und Wassermann, 1913), and since Trichomonasis has not been reported in chicks, the name of *Trichomonas pullorum* N. Sp. is proposed by the writer.

SOURCE OF INFECTION

The source of the infection was not definitely determined. Evidently it might come from the soil, the water supply or from "carriers." With incubator chicks it would seem that both the soil and the "carrier" sources can be eliminated, thus leaving the water supply as the probable source.

CONTROL OF THE DISEASE

If the source of the organism is in the water supply its elimination can be effected by boiling or by disinfectants. No opportunity was afforded for trying out these means. By chance another method of control was, however, discovered. One lot of very sick chicks was brought to the laboratory and fed on sour milk. These made a slow recovery. Subsequently healthy chicks were infected in the laboratory and developed the disease; they were then removed from the pens, and their lives saved—if the disease was not too far advanced,—by feeding sour milk. It is quite likely that the lactic acid in the milk acts as an antiseptic or even as a germicide. Possibly other substances could be found equally efficacious, but the use of

sour milk appears to be both rational and convenient, and at the same time it serves as an excellent food.

In closing, the writer desires to acknowledge the receipt of valuable aid concerning the literature, from Dr. C. A. Kofoid, University of California, Dr. E. J. Lund, University of Minnesota, and Dr. A. Eichhorn, Bureau of Animal Industry Washington, D. C.

A STUDY OF THE DIPHTHEROID GROUP OF ORGANISMS WITH SPECIAL REFERENCE TO ITS RELATION TO THE STREPTOCOCCI[1]

PART III. IMMUNOLOGICAL RELATIONS

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IMMUNITY

Natural immunity

Both man and animals possess a relatively high degree of immunity against infection by this group of organisms. Bergey (1904), however, reports spontaneous abscesses in guinea-pigs caused by a diphtheroid, and Klein (1903) proves the causal relationship of this organism to pneumonia in rats. The bacillus of Preiz-Nocard (*B. flavidus*) is pathogenic for most of the lower animals and frequently causes infection among them.

Artificial immunity

Hamilton and Ruediger (1904) were able to develop protective sera against *B. Ruedigeri.* Hall and Stone (1916) report a partial neutralization of the soluble toxin of the Preiz-Nocard organism by diphtheria antitoxin. One minimal ethal dose of their *B. flavidus* toxin required two hundred and fifty times as much diphtheria antitoxin to neutralize it as is required by one minimal lethal dose of the Klebs-Loeffler bacillus toxin. They suggest a group reaction among toxins like the similar reactions which exist among agglutinins. I have been able to develop an antiserum with a filtrate of my strain 1 which pro-

[1] A Thesis for the degree of Doctor of Public Health.

tected against its characteristic effects (Part I). Hamilton (1907) and Rosenow (1915c) report cases which were very markedly benefited by vaccines of diphtheroid bacilli. In Hamilton's cases the vaccines produced a marked rise of the opsonic substances in the serum. On the other hand, vaccines have produced no consistently good results in Hodgkin's disease. Bloomfield gave as many as 10,000,000,000 of the killed bacilli without as much as producing a local reaction. Likewise Townsend's case of diphtheroid cystitis did not seem to be greatly benefited by vaccines.

Phagocytosis

There is very little direct information on this subject, although from the predominance of the purulent conditions produced by this group it is safe to assume a very marked phagocytic response. Experimental infections with my strain 1 developed phagocytosis in some organs but not in others. It was most marked in the joints, while in the gall bladder and kidney almost none could be seen. There was phagocytosis by the splenocytes and in the endothelial-celled thrombi formed in the walls of the blood and lymph vessels of rabbits. Teacher (1915) notes a phagocytosis of *B. flavidus* by neutrophiles present in the damaged placenta of his guinea-pigs.

Toxin formation

It is exceptional for members of this group to form soluble toxins. Ruediger (1903) and Hamilton (1904) have isolated diphtheroid bacilli from both normal and pathological throats which produced a soluble toxin that quickly killed guinea-pigs. Animals were not protected with diphtheria antitoxin, but were protected by a homologous antitoxin. Hektoen was able to demonstrate toxic substances in the cultures of his diphtheroid which produced lesions similiar to those produced by a live culture, although they rarely caused death. Diphtheria antitoxin did not protect. Hall and Stone (1916) report of their *B. flavidus* "1 cc. of unaltered culture kills in fifteen hours. Ten cubic centimeters of the supernatant passed through a Berkefeld filter kills in fifteen hours. One cubic centimeter of toxin-

free culture is fatal in twenty-eight days. The strongest toxin developed killed a guinea-pig in forty-eight hours in a dose of 0.2 cc." Strain 1 of my series produced a soluble toxin in twenty-four hour broth filtrates. It was neutralized by a specific antiserum (Part 1).

Hemolysins

Hall and Stone (1916) report the development of a hemolysin in their cultures, which was not lost on cultivation and was not destroyed by ten minutes boiling. According to Fox's (1915b) tabular reports several of his cultures give positive hemolysis. Strains 13 and 14 of my series gave a slight hemolysis which was lost on continued cultivation.

Agglutination

There have been relatively few agglutination experiments performed with the diphtheroid group. The vast majority of them have attempted to establish a relation between the pseudodiphtheria bacillus (*B. Hoffmannii*) and the true diphtheria bacillus.

Luebowski (1900) developed an immune serum in a goat by using non-virulent diphtheria bacilli. This anti-serum agglutinated twenty-three strains of virulent and two strains of avirulent diphtheria bacilli. The positive dilutions of the serum ranged from 1-80 to 1-160. Three strains of *B. Hoffmannii* were negative.

Lesieur (1901) obtained an anti-serum with diphtheria bacilli and tested 40 strains of Klebs-Loeffler and 30 strains of the pseudodiphtheria organism against it. Of the 70 strains, 29 virulent and 21 avirulent strains reacted negatively, while the remainder were positive. He considers that this is strong evidence in favor of the relationship of certain strains of each series. It is also evidence that the strain variation is quite marked.

Schwoner (1902) obtained a powerful anti-serum in a horse by starting the inoculations with dead cultures and ending with living diphtheria organisms; 50 strains of the Klebs-Loeffler

bacillus reacted positively in dilutions of the serum varying from 500 to 10,000. Reactions with pseudo-diphtheria strains were negative. A goat immunized against a pseudo-diphtheria organism agglutinated only the homologous strain, having no effect on 4 other strains of pseudo-diphtheria bacilli. He concluded from this and similiar experiments that there were many different strains of the Hoffmann bacillus.

Lipstein (1908) developed agglutinating sera in rabbits and found a marked strain specificity. Hamilton (1907) separates three groups of pseudo-diphtheria by means of agglutination reactions. Group I is the common *B. Hoagii*; Group II, *B. flavidus* and Group III the Ruediger bacillus. She says that the agglutinating as well as the lytic sera for these types are specific.

Torrey (1916) has isolated an anaerobe from lymph glands (mostly Hodgkin's) which he believes to be a distinct species, as proved by agglutination experiments.

Teoumin (1913) claims to have separated four groups of pseudo-diphtheria bacilli by agglutination experiments. He developed a 1-400 titre following the third injection. The homologous strains agglutinated, but there was no cross reaction.

Agglutination experiments

For this work I have chosen several strains representative of different subgroups of the diphtheroids. Preliminary experiments in this line correlated with the work of others convinced me that it was not advisable to seek by this criterion an ultimate means of classification. The work done is therefore not as elaborate as it would have been if the phenomenon had been more promising for the purpose for which I wished to employ it.

There are certain definite ends, however, which I thought could be served, and briefly put they are as follows: first, I aimed to get some notion of the relation of my pathogenic strains to the more saphrophytic members of the group; second, to verify my hypothesis regarding the biologic relationship of certain streptococci to certain diphtheroids; and third, to show the bio-

chemical identity of strain 1 in its coccus form with the barred type of the bacillus which gave rise to it.

The technique employed was as follows: The serum dilutions were made in small test tubes, each of which contained 1 cc. of 0.8% salt solution. To these tubes was added 1 cc. of a twenty-four hour broth culture of the organism. The number of organisms in the culture used was held fairly constant by comparison with an arbitrary standard of turbidity. In case the culture was clumped it was put in a mechanical shaker for twenty-four hours. The different dilutions were incubated at 37°C. for one to two hours, placed in the ice-chest over night and final readings taken after eighteen to twenty-four hours. All readings were macroscopic. Dilutions of normal rabbit's serum constituted one set of controls, but in addition a tube of 0.8% NaCl plus 1 cc. of the twenty-four hour broth culture was also used. The various grades of reaction were distinguished by the number of *plus* marks opposite the dilution. *Four plus* indicates complete agglutination with a perfectly clear supernatant liquid: *three plus* indicates a slight turbidity in the supernatant: *two plus* approximately 50 per cent of the organisms present at the bottom of the tube as a sediment: *plus one* indicates only a slight precipitation and *sl* a slight clumping insufficient to cause appreciable precipitation.

Recourse to tables 4 and 5 shows the agglutination reactions of serum 1 with some of the other strains used as antigens. As might be expected the homologous antigen gave a fairly high titre, the reaction being quite noticeable in the 1–2560 dilution, and almost complete in the dilution of 1–1280. The other strains showing a powerful sugar fermentation are strains 14, 16, 11, 2 and 3. It may readily be seen that the bulk of cross-agglutination occurs with these strains, with the exception of strain 11. Strain 12 is *B. xerosis* and T-337 is *B. flavidus*, both of which are mutually related morphologically and fermentatively. They show about the same amount of cross reaction, in low dilution only. Strains 10 and 31 are *B. Hoffmannii*, and give only slightly less cross reaction than strains 10 and 12. Strain 4 is a variant of *B. Hoagii*, which failed to ferment maltose.

It is evident that the active fermenters form a group by themselves, while between the rest of the different types used less demarkation is apparent.

Controls with the various antigens in dilutions of 1–20 normal serum were only slightly positive, and in all cases but one were

TABLE 4

STRAIN NO.	DILUTIONS OF IMMUNE SERUM								IMMUNE SERUM
	1–20	1–40	1–80	1–160	1–320	1–640	1–1280	1–2560	
J 1	++++	++++	++++	++++	+++	+++	+++	++	I
2	+++	++	+	++	++	+			I
3	++++	++++	+++	++	+++	+	±		I
4	+++	++	++	+					I
10	+++	+++	+	+					I
11	+++	++	+	+					I
12	+++	+++	++	+	−	−	−	−	I
14	++++	+++	++	++	+	+	+		I
16	++++	++++	+++	+++	++	+	+		I
T337	+++	+++	++	++	+				I
31	+++	++	+	+					I

TABLE 5

STRAIN NO.	DILUTIONS OF NORMAL SERUM								CONTROL 0.8 NaCl PLUS 1 CC. CULTURE
	1–20	1–40	1–80	1–160	1–320	1–640	1–1280	1–2560	
J 1	+								−
2	++								−
3	++++	++	++						−
4	++								−
10	++								−
11	++								−
12	+								−
14	+								−
16	+								−
T337	+								−
31	++								−

completely negative in 1-40 dilutions. Controls with 0.8% NaCl were negative.

It will be necessary to make some explanation regarding immune serum VI. After three injections of the germ-free fil-

trate the animal in question received on December 4, 1915, 1.5 cc. of a twenty-four hour agar culture of live organisms of strain 1 in NaCl solution intravenously. On December 11, 1915, it received 1.75 cc. and on December 15, it received 2 cc. The agglutination titre on December 21 was + + + in a dilution

TABLE 6

STRAIN NO.	DILUTIONS OF IMMUNE SERUM								IMMUNE SERUM NO.
	1–20	1–40	1–80	1–160	1–320	1–640	1–1280	1–2560	
Bile 43..	++++	++++	++++	++++	+++	+++	+++	++	I
Bile 43....	–	–	–	–	–	–	–	–	VI
J1.........	++++	++++	++++	++++	+++	+++	+++	++	I
J1.........	–	–	–	–	–	–	–	–	VI
Lewis streptococcus...	+++	+++	+++	++	++	+			I
Lewis streptococcus...	+++	+++	+++	++	++	++			VI
16.........	+++	++	++	++	++	+++			VI
Joints 43..	++++	++++	++++	++++	+++	+++	+++	++	I
Joints 43..	–	–	–	–	–	–	–	–	VI
J1..	+	–	–	–	–	–	–	–	β streptococcus
Robert's streptococcus...	+	–	–	–	–	–	–	–	I
Beta streptococcus	+	–	–	–	–	–	–	–	VI
Robert's streptococcus...	+	–	–	–	–	–	–	–	VI
T337......	+++	+++	++	++					VI
31.........	++	++	++	+					VI

of 1–640. On December 23 the animal received 4 cc. of a forty-eight hour agar culture, but on testing the serum on January 4, 1916, there was absolutely no agglutination with the homologous strain. Just why this rabbit VI which had reacted positively should after another large dose react negatively I am at

loss to explain satisfactorily, although a fuller discussion of this will be entered into later.

It is very curious that, although this serum lost its agglutinating power for the homologenous strain, it still gave cross agglutinating reactions. (See antigens 16, 31, and T337). I was able to make use of this aberrant phenomenon in the demonstration of the identity of the coccic and bacillary forms of the organism, as is shown in table 6.

Recourse to table 1 (Journal of Bacteriology, **2**, 99) shows that rabbit 43 received intravenous injections of strain 1 in bacillary form, and from the gall bladder the organism was recovered in long chains of streptococci or diplostreptococci. In the joints, blood and other organs only diplococci were found. I interpreted this to be an instance of extreme pleomorphism and attempted to test my hypothesis by immune reactions.

If serum VI failed to agglutinate the homologous strain, although it still cross agglutinated related strains, it is obvious that a negative reaction with the organisms recovered from this animal would be strong evidence that the injected bacillus was able to grow in long chains of streptococci. Furthermore, if these streptococcoid forms were agglutinated by homologous serum I to the same extent as the organism which gave rise to this serum, the evidence for the identity of these organisms of such diverse morphology would be still stronger.

Reference to the agglutination tables 4 and 6 will show that just this series of reactions did occur. Bile 43 antigen, which is prepared from the streptococcus form recovered from the bile of the animal injected with the diphtheroid bacillus I, is attacked to the same degree by serum I as is its homologous antigen (I). On the contrary, the reaction with serum VI is entirely negative. The same result is seen with the diplococci recovered from the joints, which antigen is designated "Joints 43." It will also be seen that serum VI still shows cross reactions with antigens 16, T–337 and 31 to about the same extent as was shown by serum I under table 4.

Table 6 also shows some other very interesting reactions. In

addition to the cross reactions of serum VI with other members of the diphtheroid group, a very definite agglutination with the non-hemolytic streptococcus (Lewis) also occurs. Serum I also gives the same reactions to the streptococcus. On the contrary, other types of streptococci give absolutely no cross reactions. The strain "Roberts" which is a hemolyzer of alpha type (*S. viridans*) reacts with neither serum I nor VI. Likewise the streptococcus of beta type of hemolysis (Smith (1915)) gives no reaction with serum VI. Conversely, its immune serum does not react with antigen I, although it reacts feebly with the strain, "Lewis streptococcus." This highly suggestive evidence of the relationship between the streptococcus and the diphtheroid groups should be qualified by stating that it is very probable that the contact is made at certain definite points only through the medium of special strains such as we have here, and does not necessarily imply a relation at other points. For example, the serum labeled beta streptococcus was highly agglutinative for its homologous antigen, but did not agglutinate strain 1 in even a 1–20 dilution. It did, however, give a mild agglutination in 1-40 with the non-hemolytic streptococcus. Likewise the alpha type of streptococcus which is an incomplete hemolyzer gave negative reactions with both serums I and VI.

One sees here a diphtheroid strain not only giving cross reaction with other strains of the same group, but also giving a very definite reaction with a proved streptococcus, and in most cases in greater dilutions than with the diphtheroids. Such crossing is not indiscrimate, however, inasmuch as it does not extend to the representative types of the hemolytic streptococci.

Further experiments with serum VI

I have already referred to the peculiarity developed in this serum, viz., its negative response to the homologous antigen and the positive response to heterologous antigens. It occurred to me that the large doses of living cultures of a fairly active organism might have paralyzed the agglutinating response, although the fact that some antigens did react was rather opposed to such a contention. In case this hypothesis could not be

validated, it occurred to me that the electrical conditions which are supposed to be responsible for the actual agglutination might have been disturbed. To this end I employed a bivalent ion ($CaCl_2$) with the hope that the stronger charge carried by the Ca ion would finally succeed in agglutinating the organisms, provided that they had been actually sensitized (in the sense of Bordet). In place of NaCl as the diluting solution, I used $CaCl_2$, but the results did not vary. I then tried an absorption experiment on serum VI. I mixed a very heavy emulsion of the organism with a 1–40 and 1–160 dilution of the serum, giving ample opportunity for sensitization of the bacilli. On centrifugation, pipetting off the supernatant fluid and

TABLE 7

STRAIN NO.	DILUTIONS OF IMMUNE SERUM								IMMUNE SERUM	DATE
	1–20	1–40	1–80	1–160	1–320	1–640	1–1280	1–2560		
J381...	++++	++++	++++	+++	++	++	++	+	VI freshly drawn	2,11,16
J1.....	+	–	–	–	–	–	–	–	VI	
16.....	+++	++	++	++	++	+++			VI	
J1 in CaCl	+	–	–	–	–	–	–	–	VI	

treating with strain 16, I was able to get the same agglutination as with the serum which had not been exposed to the hemologous antigen. This experiment proved that no sensitization of the bacilli took place, and explained the failure of the $CaCl_2$ experiment.

This animal, VI, received its last dose of live organisms on January 1, 1916, and on January 14, 1916, the serum was again tested with the same results. This serum was placed in the icebox and on February 9, 1916, was tested against a bacillary form of strain 1, which had been obtained from the diplococcus form. Surprising as it may seem, this organism (J-381) tested out on February 11, 1916, on serum VI drawn January 14, was agglutinated as strongly as previously by serum I. Moreover, ruling out the effect of the low temperature for three weeks, by

using a fresh specimen of the serum, the same action took place, although it was still refractory to the original strain in diplococcus form.

Controls in 0.8% NaCl and normal serum were all negative. It would seem very probable that some chemical change took place in the organism as a result of the conditions necessary to bring it back to the barred diphtheroid form. There is other evidence for this contention. The bacillary form of this organism is acid-fast to 2 per cent HCl in 20 per cent ethyl alcohol. When converted to the diplococcus form it is non-acid fast, indicating a change which is more than merely one of shape.

My only justification for reporting these results lies first in the fact that I have used this aberrant phenomenon as an aid in demonstrating a rather unusual contention, and second to exhibit one of the freakish occurrences which so many have experienced with the agglutination reaction from time to time.

Complement fixation

There have been but few observations made on the diphtheroid group or on the Klebs-Loeffler bacillus by means of the complement-fixation test. Morse (1912b) reviews what little literature has appeared on the subject, and I shall quote largely from her paper. Lambotte (1901) produced strong specific sera in guinea-pigs for the diphtheria bacillus, a pseudo-diphtheria bacillus from the normal human conjunctiva, and also one from a membrane on a fowl's eye. He gives no description of these bacilli. He shows cross-reactions between the anti-diphtheroid sera and the diphtheria bacillus. Kolmer (1912) has done a convincing piece of work on this subject, using eight strains from diverse members of the diphtheria group, including the Klebs-Loeffler bacillus and *B. Hoffmannii*. He studied several strains of each bacillus representing the different types of bacilli according to Wesbrook's classification, and selected them from different sources. His work tends strongly to show that there is a relationship between *B. Hoffmannii* and the diphtheria bacillus.

On the other hand, Morse is unable to discover any such relation, using the same general method. She says in part: "There are no reciprocal complement-fixation reactions between the typical diphtheria bacillus and representatives of the four species of the diphtheroid group." However, one does not get very definite notions from a careful scrutiny of her experiments. She used only five organisms, and was not successful in developing anti-sera with two of them. Very few of her end results show an appreciable margin between the fixing powers of normal and immune sera. A study of the literature on agglutination work in this group shows that there is probably a great diversity of strains of *B. Hoffmannii* and of the Klebs-Loeffler bacillus. Therefore it would be difficult to draw any conclusions from negative results with so few organisms as Morse used. Priestly (1911) tried to use this reaction for the differentiation of the diphtheria bacillus and diphtheroids, but could develop no satisfactory antigens. He used bacterial extracts and suspensions, but neither proved efficient. Armand-Delille (1908) used diphtheria toxin as antigen, but found wide individual differences in the fixing power of the sera which he could inversely correlate with the signs of anaphylaxis during the immunization. Poujol and Delance (1908) confirmed his results. Cathoire (1911) showed that the serum of healthy carriers fixed complement in the presence of diphtheria toxin.

Hamilton (1907) has developed specific lytic sera for three groups of pseudo-diphtheria bacilli. She specifies the Ruediger bacillus as one, while the others have been identified as *B. Hoagii* and *B. flavidus*, in Morse's classification study.

Technique

1. Antigen preparation. In the preparation of this cardinal component of the system used in complement-fixation work, the method of Swift and Thro (1911) was followed with very slight variations. (a) The organisms were grown on the surface of plain or glucose agar in large Petri dishes for from two to five or six days. The smears were always examined for their purity.

(b) Growth was washed off with sterile 0.85% NaCl and strained if admixed with any of the media. (c) Emulsions were centrifuged at high speed, and organisms washed at least twice with sterile NaCl. The supernatant fluid was pipetted off and the organisms desiccated in vacuo over H_2SO_4 in the icechest (d) The dried bacilli were thoroughly ground with fine sea-sand which had been incinerated to free it of any organic material; then shaken for forty-eight hours in 0.85% NaCl solution containing 0.35% phenol. (5) The débris was then thrown down in a high speed centrifuge and the supernatant fluid stored in dark glass bottles in the icechest. This was kept for the stock extract, dilutions being made up as needed.

2. Complement. Pooled serum of five or six guinea-pigs was diluted to 10 per cent. Graded amounts of a 5 percent solution were titrated against a constant quantity of anti-sheep hemolysin and 5 percent solution of sheep's corpuscles. In the final tests 2 units of complement combined with 0.5 cc. of 1-1000 dilution of anti-sheep amboceptor.

3. Corpuscle suspension. Five percent suspension of sheep's erythrocytes, used in amounts of 0.5 cc. to a tube.

4. Immune sera. Half-grown rabbits were chosen for this purpose. The non-specific fixing powers of a certain percentage of normal rabbit's serum has been shown by Kolmer (1916) and others. Preliminary anti-complementary tests were performed on the rabbits used in this work, and only those selected whose sera reacted negatively with the antigen used. Kolmer has also emphasized another important point in this connection, viz., that any slight tendency a rabbit's serum may have to yield non-specific fixing bodies with bacterial antigens is greatly increased, by exposure to a temperature of 56°C. for a half hour. If the temperature is kept at 62°C. this tendency is much decreased, while it is entirely removed by heating at 70°C. He has also demonstrated that 62°C. does not impair to any significant extent the immune bodies in the serum. Consequently all my sera were inactivated at 62°C., instead of 55°C.

It required from four to six weekly injections of moderately large doses of the living organisms to develop the fixing power

that I desired in the sera. The organisms were grown on glucose agar.

It was the original intention to develop eight immune sera with as many different strains of organisms which seemed fairly representative of the series studied. All animals yielded a satisfactory serum except the ones receiving strain T337. These developed a progressive loss of weight until one-half of their original weight had been lost. Their sera yielded no immune bodies, and they usually died at this stage. This experience is in accord with that of Hamilton (1907) when working with *B. flavidus*.

Results of experiments

The anti-sheep hemolytic system was used in all the tests. After determining the anti-complementary dose of the antigen it was used in one-fourth to one-sixth of that quantity. The preliminary test for the strength of the components of the hemolytic system was performed as follows: 0.5 cc. of a 5 percent suspension of fresh sheep's corpuscles was added to 0.5 cc. of an amboceptor diluted 1–1000 in NaCl. A 5 percent solution of pooled complement was used in graduated amounts, from 0.5 cc. to 0.1 cc. NaCl solution was added to bring the volume to 2.5 cc. The time limit was thirty minutes. Simultaneously an amboceptor control was run as follows: 0.5 cc. of the corpuscles plus 1 cc. of 5 percent complement with graded amounts of amboceptor from 0.4 to 0.1 cc. In case complete hemolysis did not develop in the last two tubes, the amboceptor was not considered to be too strong.

Complement and antigen were mixed with NaCl to make a total volume of 1.5 cc., added to each tube containing the immune serum, and incubated for forty minutes. Simultaneously the necessary quantity of sheep's cells and amboceptor were mixed and incubated for forty minutes for sensitization. At the end of this time 1 cc. of this mixture was added to each tube of the complex and readings taken at the end of one hour.

In the fixation work shown in the following tables (8 to 18 inclusive) the amount of immune serum used was at no time

greater than 0.1 cc., the reason for this procedure being given more in detail on page 470. An inspection of the tables will show that cross-fixation failed in many instances when using 0.1 cc. of the serum. With antigen T337 almost none of the sera gave cross fixation. Antigen 12 was little better. It might be argued that strain T337 had absolutely no relation to the other diphtheroid races. As a matter of fact such is not the case, and in order that misconceptions of this sort may not arise, and that the true significance of this negative reaction may be interpreted, I concluded to carry the experiments further.

When increasing amounts of immune serum are employed (table 21) the antigen T337 exhibits very definite cross-fixation with several strains, and there is still a margin between a normal and immune serum. Strain T337 by its fixation reaction would seem farther removed from most of the diphtheroid strains than they are from one another; but it is not removed in the direction of the streptococcus group, as is strain 1, but, from a consideration of its cultural and other characteristics, we have good reason for believing, in the direction of the Klebs-Loeffler bacillus itself.

That the use of these increased amounts of immune serum shown in table 21 is legitimate, can be seen from the results in table 19; it is evident that antigen T337, as well as several others, gives no reaction with normal heated serum until it is used in quantities of about 0.25 cc., and that then the fixation is only slight. This circumstance permits the use of immune sera in amounts just short of those which will give fixation with normal sera. Accordingly in table 21 fixation experiments have been performed using graded amounts of immune serum, until 0.25 cc. is reached. The sera and antigens which gave no interreaction in the tables 8 to 18 were naturally selected.

TABLE 8

Table showing fixing and anticomplementary power of antigens

NORMAL SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	T-337	X-323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.1	0.1	C.H.	C.H.	C.H.	S.H.I.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.15	C.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.2	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.3	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	I.H.	C.H.	S.I.H.	M.I.H.	C.H.
0.1	0.35	M.I.H.	C.H.	C.H.	I.H.	C.H.	C.H.	I.H.	C.H.	M.I.H.	I.H.	M.I.H.
0.1	0.4	I.H.	C.H.	C.H.	I.H.	S.I.H.	M.I.H.	I.H.	M.I.H.	I.H.	I.H.	I.H.
0.1	0.5	I.H.	M.I.H.	M.I.H.	I.H.	M.I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.
Antigenic unit...		0.07 cc.	0.09	0.08	0.02	0.08	0.07	0.03	0.07	0.055	0.05	0.065
Immune serum												
0.1	0.01	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.				C.H.
0.1	0.03	I.H.	I.H.	I.H.	S.I.H.	I.H.	I.H.	I.H.				M.I.H.
0.1	0.05	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.
0.1	0.07	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.
0.1	0.09	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.
0.1	0.12	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.
0.1	0.15	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.	I.H.				I.H.

The following abbreviations will be used in all tables to record reactions: C.H., complete hemolysis; S.I.H., slight inhibition of hemolysis, 10 to 20 per cent; M.I.H., marked inhibition of hemolysis, 75 to 90 per cent; P.H., partial hemolysis, approximately 40 to 50 per cent; I.H., complete inhibition of hemolysis.

TABLE 9

Immune serum 1

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	T-337	X-323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	M.I.H.	C.H.	C.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.03	com-	I.H.	C.H.	C.H.	M.I.H.	P.H.	I.H.	M.I.H.	C.H.	M.I.H.	C.H.	C.H.
0.05	ple-	I.H.	C.H.	C.H.	I.H.	M.I.H.	I.H.	M.I.H.	C.H.	I.H.	C.H.	C.H.
0.01	men-	I.H.	C.H.	C.H.	I.H.	M.I.H.	I.H.	M.I.H.	C.H.	I.H.	C.H.	C.H.
0.01	tary	I.H.	S.I.H.	C.H.	I.H.	I.H.	I.H.	M.I.H.	C.H.	I.H.	C.H.	C.H.
	dose											

TABLE 10

Immune serum 2

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	T-337	X-323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	S.I.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.03	com-	C.H.	I.H.	I.H.	S.I.H.	M.I.H.	S.I.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.05	ple-	C.H.	I.H.	I.H.	I.H.	S.I.H.	I.H.	S.I.H.	C.H.	P.H.	C.H.	C.H.
0.07	men-	C.H.	I.H.	I.H.	I.H.	M.I.H.	I.H.	S.I.H.	C.H.	P.H.	C.H.	C.H.
0.1	tary	C.H.	I.H.	I.H.	I.H.	M.I.H.	I.H.	P.H.	C.H.	P.H.	C.H.	S.I.H.
	dose											

TABLE 11

Immune serum 34

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	S.I.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03	com-	C.H.	I.H.	I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	ple-	C.H.	I.H.	I.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.07	men-	C.H.	I.H.	I.H.	C.H.	C.H.	I.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.1	tary dose	S.I.H.	I.H.	I.H.	C.H.	C.H.	I.H.	S.I.H.	C.H.	S.I.H.	C.H.	S.I.H.

TABLE 12

Immune serum 32

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03	com-	C.H.	S.I.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	ple-	S.I.H.	M.I.H.	C.H.	I.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.07	men-	P.H.	M.I.H.	M.I.H.	I.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	tary dose	P.H.	S.I.H.	I.H.	I.H.	I.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.

TABLE 13

Immune serum 31

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6 Anti-complementary dose	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01		C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03		C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05		S.I.H.	M.I.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.
0.07		I.H.	M.I.H.	C.H.	C.H.	I.H.	S.I.H.	C.H.	S.I.H.	S.I.H.	C.H.	S.I.H.
0.1		I.H.	M.I.H.	C.H.	C.H.	I.H.	S.I.H.	C.H.	M.I.H.	S.I.H.	C.H.	M.I.H.

TABLE 14

Immune serum 3

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6 Anti-complementary dose	C.H.	I.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01		M.I.H.	I.H.	I.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03		I.H.	I.H.	I.H.	S.I.H.	C.H.	I.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.
0.05		I.H.	I.H.	I.H.	M.I.H.	C.H.	I.H.	S.I.H.	S.I.H.	C.H.	C.H.	C.H.
0.07		I.H.	I.H.	I.H.	M.I.H.	C.H.	I.H.	S.I.H.	M.I.H.	S.I.H.	P.H.	C.H.
0.1		I.H.	I.H.	I.H.	M.I.H.	C.H.	I.H.	P.H.	M.I.H.	M.I.H.	M.I.H.	S.I.H.

TABLE 15

Immune serum streptococcus 20 (Lewis)

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.
0.03	com-	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.
0.05	ple-	P.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	C.H.	C.H.	C.H.
0.07	men-	P.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	S.I.H.	P.H.	C.H.
0.1	tary	M.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.	C.H.	M.I.H.	M.I.H.	C.H.
	dose											

TABLE 16

Immune serum 12

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	337	323	H. STREPTOCOCCUS	12
cc.	*cc.*											
0.005	1–6	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.
0.03	com-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	I.H.
0.05	ple-	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	I.H.
0.07	men-	C.H.	C.H.	C.H.	C.H.	S.I.H.	C.H.	C.H.	P.H.	C.H.	C.H.	I.H.
0.1	tary	C.H.	C.H.	P.H.	P.H.	P.H.	S.I.H.	C.H.	M.I.H.	S.I.H.	C.H.	I.H.

TABLE 17

Antigen 1 against various sera

IMMUNE SERUM	AMOUNT ANTIGEN	SERUM 1	SERUM 2	34	32	31	3	LEWIS STREPTOCOCCUS 20	12
cc.	*cc.*								
0.005	1–16	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.01	Anti-	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.03	comple-	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.05	mentary	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	P.H.	C.H.
0.07	dose	I.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	M.I.H.	C.H.
0.1		I.H.	C.H.	C.H.	M.I.H.	C.H.	M.I.H.	I.H.	C.H.

17A

Antigen from bile of rabbit 4

IMMUNE SERUM	AMOUNT ANTIGEN	SERUM 1
0.005	1–6	C.H.
0.01	Anti-	M.I.H.
0.03	comple-	I.H.
0.05	mentary	I.H.
0.07	dose	I.H.
0.1		I.H.

TABLE 18

Antigen 1 against various sera

IMMUNE SERA	AMOUNT ANTIGEN	ANTIGEN 1	2	34	32	31	3	LEWIS STREPTOCOCCUS 20	H. STREPTOCOCCUS		
cc.	*cc.*										
0.005	1–16	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		
0.01	Anti-com-	S.I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		
0.03	plemen-	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		
0.05	tary dose	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.		
0.07		I.H.	C.H.	C.H.	C.H.	C.H.	S.I.H.	M.I.H.	C.H.		
0.1		I.H.	C.H.	C.H.	M.I.H.	C.H.	M.I.H.	M.I.H.	C.H.		

TABLE 19

Concentration of fixing bodies in normal sera

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 34	T-337	2	3
cc.	*cc.*				
0.13	1–4	C.H.	C.H.	C.H.	C.H.
0.18	Anti-com-	C.H.	C.H.	C.H.	C.H.
0.25	plementary	S.I.H.	S.I.H.	C.H.	P.I.H.
	dose				

TABLE 20

Antigen 2 against sera

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	20	
cc.	*cc.*			
0.13	1–6	C.H.	S.I.H.	
0.18	Anti-com-	C.H.	M.I.	
0.25	plementary	P.H.	P.H.	
	dose			

TABLE 21

Antigen T-337 against sera using 1–4 anticomplementary dose

IMMUNE SERUM	1	2	34	32	31	3	20			
cc.										
0.13	P.H.	C.H.	S.I.H.	C.H.	C.H.	C.H.	C.H.			
0.18	P.H.	C.H.	M.I.H.	C.H.	C.H.	C.H.	C.H.			
0.25	M.I.H.	C.H.	M.I.H.	C.H.	S.I.H.	S.I.H.	C.H.			

TABLE 22

Antigen 34

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	31	20
cc.	*cc.*			
0.13	1–6	P.H.	S.I.H.	C.H.
0.18	Anti-comple-	P.H.	P.H.	C.H.
0.25	mentary dose	M.I.H.	M.I.H.	C.H.

TABLE 23

Antigen 3

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	32	20
cc.	*cc.*			
0.13	1–6	M.I.H.	M.I.H.	C.H.
0 18	Anti-comple-	M.I.H.	I.H.	C.H.
0.25	mentary dose	M.I.H.	I.H.	P.H.

TABLE 24

Staphylococcus antigen against various sera

IMMUNE SERUM	AMOUNT ANTIGEN	ANTIGEN 1	2	3	31	32	34	NORMAL SERUM		
cc.	*cc.*									
0 05	1–6	I.H.	C.H.	C.H.	C.H.	C.H.	C.H.	0.1	C.H.	
0.07	Anti-comple-	I.H.	C.H.	I.H.	C.H.	I.H.	C.H.	0.2	C.H.	
0.1	mentary dose	I.H.	C.H.	I.H.	C.H.	I.H.	C.H.	0.25	C.H.	
0 05	1–12	I.H.	C.H.	I.H.	C.H.	C.H.	C.H.	0.1	C.H.	
0 05	Anti-comple-	I.H.	C.H.	I.H.	C.H.	C.H.	C.H.	0.2	C.H.	
0 1	mentary dose	I.H.	C.H.	I.H.	C.H.	M.I.H.	C.H.	0 25	C.H.	

Control

That the cross fixation of diphtheroid sera is not indiscriminate can be seen by the following table, where a heterologous antigen in the form of the alcoholic extract of beef heart was employed. The results are uniformly negative.

TABLE 25

Antigen. Alcoholic extract of beef heart, reinforced with cholesterin. Antigen with syphilitic antibody in 0.1 cc.; is anti-complementary in amounts of 0.5 cc.

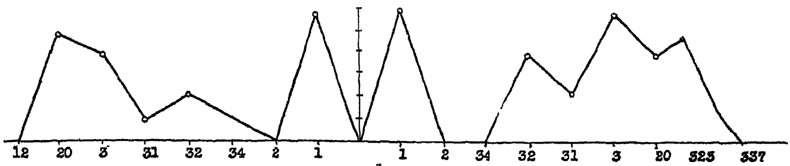
SERUM	AMOUNT ANTIGEN	1	2	34	32	31	20	3
0.05	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.09	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.1	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.15	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.2	0.1	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.	C.H.
0.25	0.1	C.H.	C.H.	C.H.	S.I.H.	C.H.	S.I.H.	C.H.

The curves which follow are attempts to formulate in a roughly quantitative manner the results shown in the preceding tables. This formulation will be presented in two phases. One curve will show the "fixing range" of the various sera toward representative antigens (right hand curve); the other will show the specific direction in which the fixing power is spent, or the so-called "elective fixations." It is obvious that these two curves are to a great extent mutual reciprocals, and the above ideas are not expressed in a segregated way by either curve alone. But since a serum involves concentration of immune bodies, and since an antigen is used mainly for specific reaction, it would seem that there is enough individualization to merit a formulation on the above basis, even though one must necessarily be expressed somewhat in terms of the other.

To this end the concentration of the immune bodies in the homologous sera was developed to as near the same titre as possible, and it will be seen that the dilutions of immune sera in the various tests were held constant and were not carried beyond 0.1 cc. There was a definite reason for using serum concentrations within a certain range. My principal object in the tests

was to determine the interrelations of various members of the group, as well as their relation to the streptococci. By using the immune serum in low concentration it was possible to bring out shades of reaction that would not have been obtained had I used sera in amounts close to the fixation level of normal sera. For this reason it will not be possible to make sweeping generaliza-

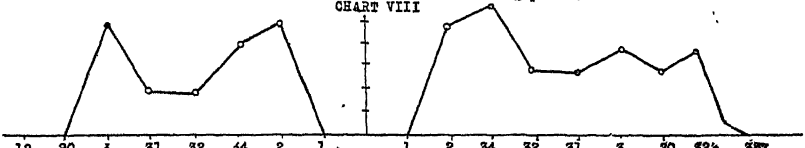
CHART VII



Antigen # 1.
Elective fixations: 1, 3, 20, 32.

Serum # 1. Fixing range
Total fixing power: 13.

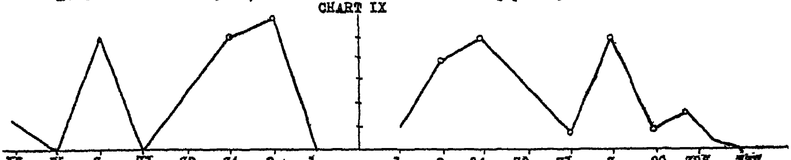
CHART VIII



Antigen # 2.
Elective fixations: 34, 3

Serum # 2 fixing range.
Total fixing power: 23.

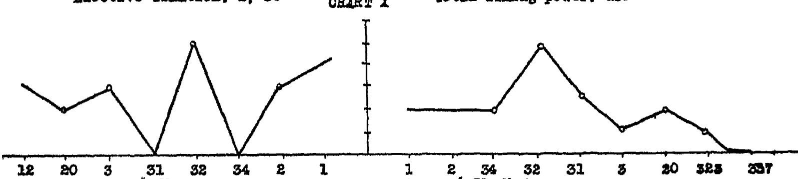
CHART IX



Antigen # 34.
Elective fixation: 2, 3.

Serum # 34 fixing range.
Total fixing power: 13.

CHART X



Antigen # 32.
Elective fixations: 1, 2, 3.

Serum # 32 fixing power.
Total fixing power: 12.

tions from these experiments as, e.g., in regard to the relation of the diphtheria bacillus to *B. Hoffmannii* which had an indirect relation to this study.

In charts VII–XIV two curves have been placed on each horizontal axis, which is divided in the center by a vertical axis. The one to the right may be called the serum curve and the one

to the left the corresponding antigen curve. The figures on the right horizontal axis represent the various antigens against which the serum was titrated, while those on the left represent the serum corresponding to the antigens against which a single antigen was titrated. The six graduations on the vertical axis represent roughly the different dilutions of sera used against the

CHART XI

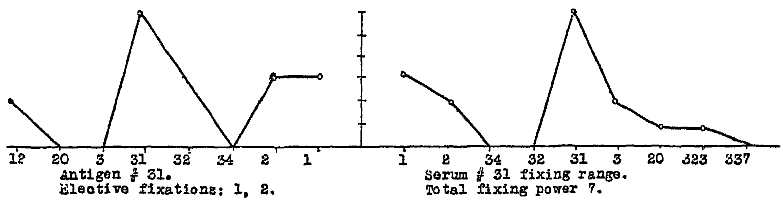


CHART XII

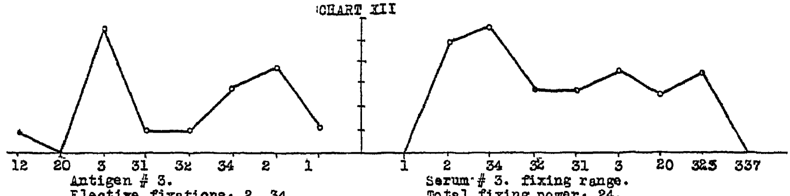


CHART XIII

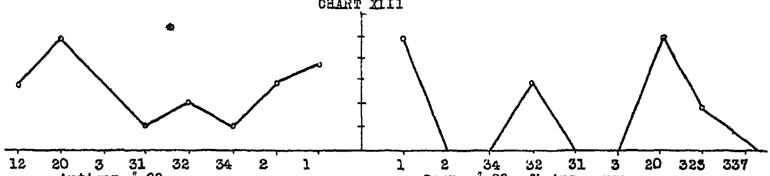
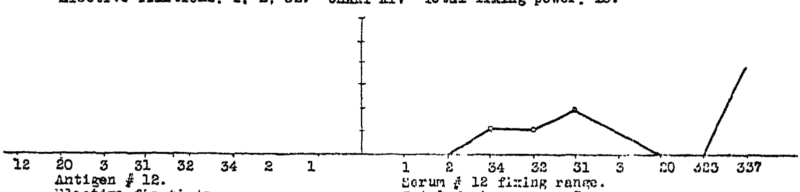


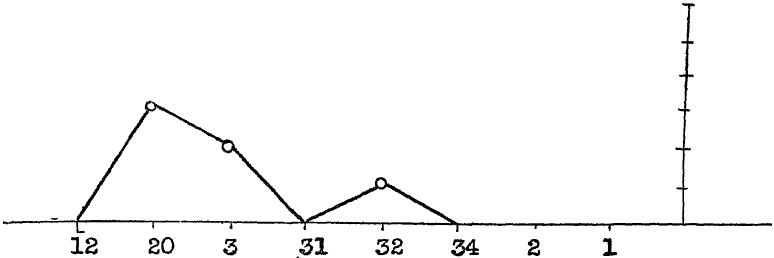
CHART XIV



various antigens. They do not in every instance record with precision what is found in the corresponding table, since the degrees of hemolysis expressed there cannot be read in per cent.

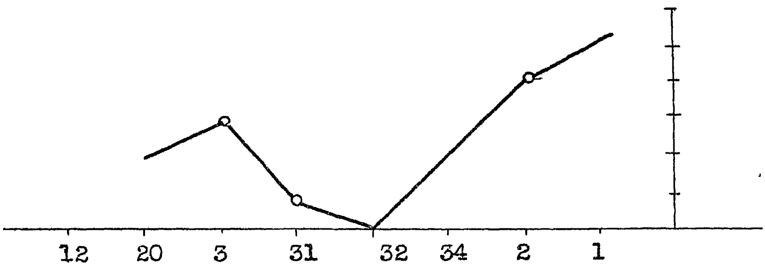
The term "fixing range" means simply the *number* of antigens which would be fixed by a certain serum, while total fixing power means an addition of all the fixations recorded which were ex-

CHART XV



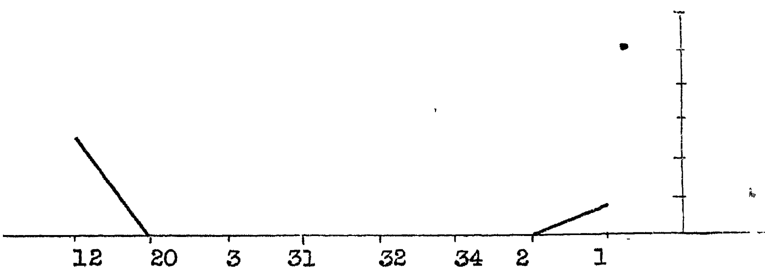
Antigen # 1. 1/16 anticomplementary dose.
Elective fixations: 20, 3, 32.

CHART XVI



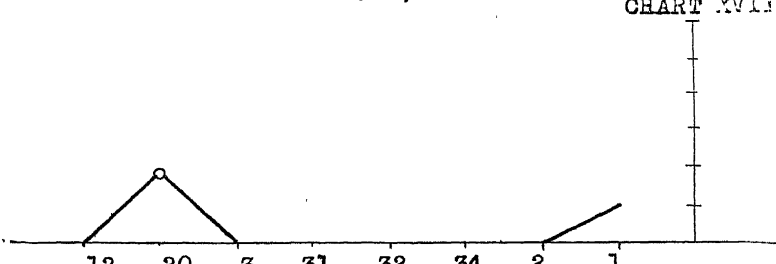
Antigen x-323.
Elective fixations: 1, 2, 3.

CHART XVII



Antigen T-337.
Elective fixations: 1, 12.

CHART XVIII



Antigen hemolytic streptococcus.
Elective fixation: 20.

pressed numerically. Each gradation on the vertical axis was given a value of 1, and on this basis the fixing power of a serum ranged from 1 to 6. The latter figure usually expressed the fixation power of the homologous serum. Certain antigens seemed to have a decided reaction with certain sera, and this was expressed by using the term "elective fixation." It is practically synonomous with cross fixation and could be called maximum cross fixation.

Discussion

We will consider first the evidence of relationship between the streptococcus and diphtheroid groups, and the part played by strain 1 in its demonstration. It will be seen from the curves that the streptococcus antigen 20 shows definite cross-fixation with the sera of strains 1, 2, and 3, but practically none at all with sera 31 and 34. In addition, serum 20 gives a strong cross reaction with antigen 1 and X-323, although the reciprocal reaction with sera 2 and 3 is not so apparent. This points to definite relation between a moderately pathogenic, non-hemolytic streptococcus, and certain diphtheroid strains. As the tables will show, this reaction is most marked in strain 1.

In order to subject this relationship to a more severe test it was decided to use a minimum quantity of the antigen. Accordingly, 1–16 instead of 1–6 of the anti-complementary dose of strain 1 was employed. Reference to table No. 18 shows the results. With the exception of the homologous serum, the streptococcus 20 gave more fixation than any other strain. In fact the rest were all negative except strains 32 and 3. This indicates a closer relation between this streptococcus and strain 1 than between the latter and all the rest of the diphtheroids used in these tests. Correlated with the other characteristics of the other two strains, it places this one in an intermediate position between the two groups and shows definitely the connection between them. However, it is probable that this contact between the groups only takes place at certain points, since it is seen that the hemolytic streptococcus antigen (H. strep.) gives no cross reaction with any of the diphtheroid strains

and only gives a slight amount with the non-hemolytic streptococcus 20.

Kolmer (1912) finds no cross-reactions between the diphtheria group and the streptococci. He studied 8 strains, with a view to showing a relation between the Klebs-Loeffler bacillus and *B. Hoffmannii.* His various antigens were tested against an anti-streptococcus serum made from scarlet fever streptococci. These were probably hemolytic, which might easily explain the negative fixation. Even so, this particular table does not appear to be controlled, inasmuch as there is no record of a normal serum being used. Since the immune serum cross-fixes in doses of 0.15 cc. and over, it would be essential to show that a normal horse serum in this small dose would also react positively to the various antigens employed.

It may next be asked what light the tests throw on the inter-relations of the diphtheroid subgroups. Scrutiny of the curves of the various sera would lead one to infer that strains 1, 2, and 3 have a similar fixing range and total fixing power. Respectively, the total fixing power for these sera is 24, 23, and 24. These might be considered as being members of the same group. This idea is further substantiated when the elective fixations of the three sera are examined. It is seen that a very close relation seems to exist between 1 and 3, and 2 and 3, but it is not so marked between 1 and 2.

It is also very noteworthy that antigen T-337 gives practically no fixation with any other serum except strain 12. Likewise the serum of strain 12 has a total fixing power of but 7, which is slightly less than that of *B. Hoffmannii.* Strain T-337 represents the subgroup *B. flavidus;* and strain 12, *B. xerosis* (Morse). Hamilton (1907) believes the former is closely related to the Klebs-Loeffler bacillus, while the latter has often been described as an avirulent diphtheria bacillus. Thus these organisms are removed biologically from the other diphtheroids, but not in the direction of the streptococcus group. The complete relation has been discussed at length under "Classification" (Journal of Bacteriology, **2**, 299). Morse (1912b) also showed an intimate relation between *B. flavidus* and *B. xerosis.* Table 21

shows its relation to *B. Hoagii*, which has also been noted by Morse, though she conceives them to be much more closely related than my experiments would indicate.

Strain 31 (*B. Hoffmannii*) has a total fixing power of only 8, although it has cross-fixations with the streptococcus group. Strains 34 and 32 have about the same total fixing power, although the former is closely related to the pathogenic group, and the latter to the saprophytic group. From table 24, it would seem that this group has a relation at certain points to the staphylococci, as sera 1 and 32 give definite cross reactions with a staphylococcus antigen, even in one-twelfth of the anticomplementary dose.

Summary

The complement-fixation reactions show that;

1. The diphtheroid group is a very complex one, the various subgroups of which may be widely removed from each other.
2. There exists an unequivocal relation between this group and the streptococci at certain points, at which points the bonds uniting the intermediate diphtheroid strain to the streptococcus group may be much firmer than those uniting it to its own group.
3. The same thing appears to be true to a much lesser degree, with staphylococci.
4. It would not be surprising if the diphtheroid group might impinge upon other more pathogenic organisms, as is connoted by the study of De Witt's strain (1912), suggesting as it does a relationship with *B. proteus*.

General pathology of diphtheroid infections

That the virulence of this group of organisms is usually not high is suggested by the fact that some member of it has been isolated repeatedly from most all of the organs of the body, whether normal or in a state of disease. When one says that most diphtheroid invasions are of the nature of sub-infections, little more comment need be made. However, there sometimes arise acute inflammatory processes which may at times be a

serious menace to life. Hodgkin's disease may begin as an acute inflammation of the lymph glands, and Bunting believes that a diphtheroid in such instances gives rise to an exudation composed of fibrin, neutrophiles, mononuclears, plasma-cells and eosinophiles. Small areas of necrosis are also produced. He claims to have reproduced the acute or toxic form of this disease in monkeys. Other examples of purulent exudation occur in otitis media which will be described under the head of "Pathogenicity" De Witt (1912), Cave (1912) and many others have described a membranous exudation arising usually from the mucosa of the throat. In De Witt's case the vagina also was involved, not only with the membrane but with an indolent ulceration as well. These organisms have produced albuminuria and sepsis (strain 2). Chronic inflammation may be produced. The interstitial changes in the lung from which strain 1 was isolated were of long standing. The fibrosis and endothelial-celled hyperplasia of Hodgkin's disease may prove to be caused by a diphtheroid.

Special pathology. It will be necessary only to refer briefly to the special pathology which has been produced by diphtheroids, as the conditions will be mentioned under the head of "Pathogenicity." Tonsillitis (Hamilton, 1904), chalazion, acute and chronic purulent otitis media (Hamilton, 1907), arthritis (Rosenow, 1915a), caseous and ulcerative lymphangitis in cattle (Hall and Stone, 1916), erythema nodosum (Rosenow, 1915b), broncho-pneumonia and interstitial-pneumonia (Rosenow, 1914b), ulcerative cystitis (Rosenow, 1915c) and an epizootic of infective abortion in guinea-pigs are the principal examples (Teacher, 1915).

Experimental pathology. Local abscess at the site of injection has been reported by many observers. Chalazion has been produced by Eyre (1896); hepatic cirrhosis by Hektoen (1901); nephritis by Dick (1915); sepsis by Hektoen (ibid), Hamilton (1904), Hall and Stone (1916), and by Rosenow (1914); paralysis by Hamilton (1904); orchitis by Hall and Stone (1916); broncho-pneumonia, myocarditis, pericarditis, erythema nodosum, cholecystitis and acute pancreatitis by Rosenow (1914b).

PATHOGENICITY

The diphtheroid group has not been taken seriously, from the standpoint of pathogenicity, by the majority of observers, although a wide divergence of opinion exists. Such an attitude has not conduced to thorough investigation and study of strains which had a suggestive connection with pathological processes. Particularly, since the claims which have been made for the diphtheroid etiology of Hodgkin's disease, those interested in the subject have usually taken a decided stand in the direction indicated by their judgment and experience. Generally speaking, the tissue pathologists pure and simple are strongly opposed to the theory of diphtheroid pathogenicity, inasmuch as they have considered Hodgkin's disease a tumor. The biologists and bacteriologists are more impressed with the pathologic possibilities of the group.

Fox (1915a) has recently made a review of the literature on this subject, and comes to the conclusion that there are only four established cases of diphtheroid infection on record. He insists strongly that the immunity reactions are the only ultimate criteria by which one is able to establish such relation, especially in view of our inability to reproduce the corresponding pathology in animals. He reviews twenty cases of a suppurative nature from the records of the Pepper Clinical Laboratory in which some type of this organism was found, and believes that endocarditis was produced in one which gave positive immunity reactions. He does not state whether immunity reactions were performed on the others. He reports three cases of pseudodiphtheritic angina which were not benefited by antitoxin. No immunity tests were recorded with these cases, although he believes the condition was caused by a diphtheroid. He concludes his paper by saying "that excepting these three cases of his own, there are but four cases of diphtheroid infection on record."

In another paper on lymph gland diphtheroids, Fox (1915b) considers the cultural, morphological and biological characteristics of various diphtheroid strains isolated from lymph glands of Hodgkins' disease and other conditions, especially enlarged

joints. In conjunction with his own strains he studied some of those from Rosenow and Kolmer. He concludes that there is no uniformity in the biology and morphology of races isolated by three observers from clinical and pathological Hodgkin's glands. Diphtheroid rods similar in biology and morphology to those found in Hodgkin's disease may be found in enlarged glands in cases of atrophic arthritis and other conditions.

I have already taken exception to the method which he employed for his sugar reactions, and have given my reasons for so doing under the head of "Fermentation" (Journal of Bacteriology, 2, 289). Close scrutiny of his morphological observations would seem to show that he varies indiscriminately the numerous factors on which a constant morphology depends. The drawings showing marked pleomorphism and staining variations are made from cultures incubated for varying lengths of time on Bordet's medium, Loeffler's blood-serum, agar, and broth arranged in such a way that there is little opportunity for drawing parallels. The advocates of morphological strain constancy all emphasize the fact that the various conditions of the experiment must be held constant in order to obtain constant results (Williams, 1902).

Wade and Harris (1915) also review the literature and come to practically the same conclusion as Fox. They believe that the diphtheroids should be regarded merely as contaminators, or at best as secondary invaders. He cites a very striking positive case of Hektoen's, and in light of it says that a few strains *may* be pathogenic. His paper is entitled "The Wide-Spread Distribution of Diphtheroids and their Occurence in Various Lesions of the Human Tissues." Since he is able to find some type or other of diphtheroids in almost every tissue examined, whether normal or diseased, he cannot attribute much pathological significance to the group.

Torrey (1916) has very recently published a study of the lymph gland bacteria. He cultured thirty strictly lymph gland conditions and ten conditions where the lymph glands were secondarily involved. He recovers one type or other of diphtheroid from twenty-two cases. His virulence experiments with

monkeys are all negative. He was able to produce nothing but transitory glandular enlargements.

Torrey recovered the pleomorphic granular type of diphtheroid in only two cases out of ten of Hodgkin's disease. He is very skeptical concerning the relation of a diphtheroid to Hodgkin's disease for the following reasons:

1. The identity of various strains has not been established.
2. Pleomorphism is common to other members of this group.
3. The description of colony formation has not been uniform.
4. There is no report of carbohydrate action of the various strains.
5. There are many types of diphtheroids reported from single cases of Hodgkin's.
6. The lack of serological evidence advanced.

In addition he says that these pleomorphic and granular forms have been isolated from a diversity of conditions. Furthermore he has investigated one of Bunting's strains from Hodgkin's glands and two of Rosenow's strains and finds no similarity of sugar or serum reactions. The morphological and cultural features also are unrelated.

He believes that considerable caution should be observed even when accepting serological results as evidence of the causal relation of an organism to a malignant growth. This surmise came as the result of his isolation of an anaerobic diphtheroid which bore no relation to the condition in which it was found, but gave strongly positive serum reactions with sarcoma and Hodgkin's cases. His critical remarks are very consistent and his position well taken.

Bloomfield (1915) also makes a study of the "Bacterial Flora of Lymphatic Glands." Examination of seven normal lymph glands showed positive cultural results in two-thirds of the cases, while a still larger per cent obtained in examination of twenty-three cases of diseased glands. He tested the virulence of 14 strains on rabbits, mice and guinea-pigs, with negative results. He believed that the organisms were parasitic but non-pathogenic. Immune reactions on patient's sera were negative except with two of the anaerobic strains. Vaccines had no effect

even in doses of 10,000,000,000 bacteria. He reports one interesting case of atypical chronic arthritis with splenomegaly, general adenitis and leucopenia. He isolated in large numbers many small Gram-positive diplobacilli; they were non-virulent for small animals, but agglutinated and fixed complement with the patient's serum. He comes to the conclusion that a great deal of conservatism should be used in passing judgment on the diphtheroid etiology of Hodgkin's disease.

I shall now briefly review those instances which I have been able to find in the literature that prove diphtheroid pathogenicity or are highly suggestive of it. At the head of this list one must place the results of Hamilton, who has always insisted that these organisms played a much larger part in disease than ever has been accredited them. Her well-reported studies are illuminating. I shall treat this subject regionally.

Eye, ear, nose and throat

In one paper Dr. Hamilton (1904) studied the virulent group of diphtheroids recovered mostly from throats of scarlet fever, measles and tonsillitis subjects. Out of 31 strains, 14 were pathogenic. The group divided itself into two classes, using as a criterion the pathology in guinea-pigs.

Class I. Intraperitoneal injection of amounts of the broth cultures equivalent to 1 per cent of the body weight of the animal gave rise to local edema, hemorrhage, peritoneal effusion, emaciation and death. Cultures from the injection site and effusion were positive. One animal died of typical post-diphtheritic paralysis in four days.

Class II. The animals died in eighteen to twenty-four hours. There was no rigor or infiltration at the injection site. Animals died of septicemia.

Eight of these 14 strains belong to Class II and are Ruediger diphtheroid bacilli. They produce a soluble toxin which is neutralized by its own antitoxin but not by diphtheria antitoxin. The other strains were pseudo-diphtheria culturally, but fermented glucose with gas. They belonged to Class I, and the animals

were protected by neither Ruediger antiserum nor diphtheria antitoxin. Here one finds at least fourteen instances of diphtheroid pathogenicity, proven by the animal test as well as serologically.

In addition the organisms in Cases number 1, 4, 9, and 13 of her series were very probably pathogenic for man, although no results were obtained in animals. Case 1 was one of acute articular rheumatism in which the organisms were found in pure culture on the tonsils. Zarniko (1889) reports a similar instance. In Case 4 a diphtheroid was obtained in pure culture from a tonsillar abscess. In Case 9 the diphtheroid was the predominating one in a severe tonsillitis of a child with an atypical rash, while in Case 13 (scarlet fever) the organism was obtained in pure culture from an aural discharge.

In another communication Hamilton (1907), she gives the results of a study of 52 cases of suppuration in various parts of the body, and 142 cases of purulent otitis media.

TABLE 26

	NUMBER OF CASES	NUMBER WITH DIPHTHERIA	PER CENT WITH DIPHTHERIA
1. Acute scarlatinal otitis media	43	31	72
2. Chronic scarlatinal otitis media	9	5	55
3. Acute non-scarlatinal otitis media	19	4	21
4. Chronic non-scarlatinal otitis media	71	11	15
5. Miscellaneous suppurations	52	11	21

In nine cases of the acute scarlatinal otitis media, the diphtheroid was found in pure culture, and in sixteen cases only a few colonies of other organisms were present. Two chronic cases with mastoid complications yielded the organism in pure culture from both locations. In the non-scarlatinal otitis media, it was found in a much smaller percentage of cases and usually mixed with many pyogenic cocci. The percentage difference between scarlatinal otitis media and between non-scarlatinal and other forms of suppuration is very marked and leads Hamilton to ask "If it is only an inhabitant of the aural tract, as has been claimed, why is it that it is found almost to the exclusion of other organisms n scarlet fever and not in other forms of otitis media?"

As further evidence of the causal relation of these organisms to this condition, she relates the results of her serological work. The gist of this work was as follows: All pseudo-diphtheritic cases showed a very markedly lowered opsonic index to this bacillus. Normal persons and those suffering from streptococcic otitis media did not show a lowered opsonic index to the pseudo-diphtheria bacillus. By injections of dead cultures of homologous strains the patient's opsonin for that strain could be raised. No ill effects followed such injections, but an apparent improvement resulted in several cases. The homologous opsonin was specific. The opsonic status could be correlated with the clinical symptoms, and the clinical condition could be predicted by a change in the index.

Ruediger (1903) found a pseudo-diphtheria bacillus in enormous numbers in seven cases of scarlatina. Agglutination reactions showed the organisms to be identical. They were highly pathogenic to guinea-pigs and produced a soluble toxin which was not neutralized by diphtheria antitoxin. This is one of the organisms also found by Hamilton (above) to be pathogenic.

Cave (1912), quoted by Fox, is firmly of the opinion that *B. Hoffmannii* gives rise to unilateral tonsillitis. The disease runs a course of one to three weeks, is associated with much irritability and a fever of 104°. The Klebs-Loeffler bacillus was absent, while *B. Hoffmannii* was found in practically pure culture. Although there was no reaction on inoculation in guinea-pigs, he did not consider that this fact disproved his contention. Hewlett, Knight, Priestly, Richmond and Salter (ibid.) substantiate the view of Cave. Beyer (1898) reports a case of pharyngitis and tracheitis due to an avirulent Klebs-Loeffler bacillus.

Spronck (1896) produced marked edema and loss of weight by injection of two diphtheroid strains into guinea-pigs. The organisms were recovered from cases of angina, from which fact he concludes that diphtheroids are the cause of certain anginas.

De Witt (1912) reports a case of suddenly-developing tonsillitis with high fever. The tonsils were so large as to almost occlude the fauces. Diphtheria antitoxin proved useless. The disease ran a septic course and in one week the patient developed

ulcerous vaginitis, and a purulent discharge from both ears. Recovery took place in six weeks, and an organism was isolated in pure culture from all lesions. It was pathogenic to guinea pigs, causing local abscess, peritoneal effusion and death. A forty-eight-hour filtered broth culture killed a pig in forty-eight hours The blood of the patient agglutinated the organism in dilution of 1–100. De Witt believes this to be a case of diphtheroid infection, and her data are very convincing.

Eyre (1896) recovered *B. xerosis* from fifteen cases of chalazion and reproduced the condition in the eyes of animals. Davis (1898) reports twelve cases of post-scarlatinal suppurative otitis media in which was found a short diphtheroid. In ten of the cases the same organism was found in the throat. One case had a membrane with no Klebs-Loeffler bacilli. The organism killed guinea-pigs in doses of 2 cc. in twenty-four hours, with general serous peritonitis, marked congestion of the organs and septicemia. Williams (1898) also isolated a similar organism from a person who had diphtheria six weeks before. It produced sepsis in guinea-pigs.

Lungs and pleura

Rosenow (1914b) reports a case of broncho-pneumonia followed by arthritis and endocarditis. From the blood of this patient he recovered a pure culture of a diphtheroid bacillus producing in rabbits pulmonary hemorrhage, broncho-pneumonia, multiple nonsuppurative arthritis, myocarditis and local infection of the muscle in the stomach. Klein (1903) reports the recovery of a diphtheroid obtained from the hepatized lung of a white rat. Inoculation of the organism into rats produced hepatization of the lung and hemorrhage. He demonstrated the organism in the tissues and recovered them in pure culture. Sepsis was also produced. My strain 26 was isolated by J. Howard Brown in pure culture from a lung abscess in a guinea-pig. He feels that it was the causative organism in this case. In another part of this paper I have demonstrated a diphtheroid to be the cause of a very curious interstitial pneumonia. (strain 1) (Journal of Bacteriology, 2, 53.)

Gastrointestinal system, liver and pancreas

Rosenow reports the isolation of a diphtheroid from acute ulcers in the stomach, in a case of acute fatal arthritis following extraction of an abscessed tooth in a patient of Dr. Billings. There were other pathological processes in the same patient from which the organism was isolated. It seemed to be the cause of the entire condition.

Hektoen (1901) has conclusively shown that a diphtheroid may produce cirrhosis of the liver. His organism was isolated from a case of blastomycotic dermatitis of the hand. In guinea-pigs it produced a cutaneous ulcer at the site of injection, and the animal died of emaciation in three to five weeks. It constantly produced necrosis and cirrhosis of the liver, and part of the time sepsis. The changes were confined to the liver in the guinea-pig. The major pathological processes were: perilobular connective tissue, proliferation with hyperplasia of the biliary passages and small areas of focal necrosis. In these lesions Hektoen demonstrated the causative organism by stain and by culture. Similar results were produced with filtered cultures, although they rarely caused death. Diphtheria antitoxin gave no protection.

It is noteworthy that subcutaneous or intravenous doses did not affect the liver in rabbits, although injection into the anterior chamber of the eye caused the typical liver lesions to develop. To a lesser extent it was possible to produce the hepatic changes in dogs, but not in the grey mouse or white rat.

Rosenow's (1915b) erythema nodosum strain has produced acute hemorrhagic pancreatitis in experimental animals.

Genito-urinary system

G. F. Dick and G. R. Dick (1915) report a series of twenty cases of acute and chronic nephritis in which they constantly found a small, pleomorphic, Gram-negative, anaerobic diphtheroid and a streptococcus. They killed laboratory animals with both of these organisms, producing albuminuria and acute

degenerative changes in the renal parenchyma. Doses of the diphtheroid as low as 2,500,000 produced headache and nausea of the same character in the patients as that from which they had been suffering. An increase of the albumin was also observed.

Francioni, quoted by Fox (1915a), describes a case of hemoglobinuria, cyanosis and jaundice without fever in which the pseudo-diphtheria bacillus was thought to be a causative factor. It was present in the blood during life, and in large numbers in all the organs after death.

Townsend (1905) reports a case of pseudo-membranous and fibrosing cystitis caused by the pseudo-diphtheria bacillus. Rosenow (1915c) isolated a diphtheroid organism from the urine in a case of ulcerative cystitis. It produced septicemia in guinea-pigs. No lesions were described. The patient's serum agglutinated specifically in high dilutions. There was no agglutination of the colon bacillus with which the organism was associated. He considers that the organism has a causative relation on account of:

"1. Its constant presence in the urine and its predominance in the smears from the scrapings.

"2. The specific and peculiar behavior of the blood toward this bacillus. The patient showed marked improvement following an injection of the dead bacilli.

"3. The high agglutinin power of the serum for this bacillus and its absence for the associated colon bacillus."

Strain 2 of my series is an anaerobic, Gram-positive, fusiform diphtheroid isolated from the blood and urine of a case of parenchymatous nephritis. Small intravenous injections produced marked albuminuria and loss of weight in rabbits. The animals finally succumbed. The evidence for the relation of this organism to the condition was therefore strong.

Teacher (1915) has isolated pure cultures of *B. flavidus* from the uterus of guinea-pigs which were the victims of an outbreak of infectious abortion. Experimentally, he has repeatedly reproduced this pnenomenon in guinea-pigs with this bacillus. He shows that the bacilli have a definite seat of election, which is the cavity of the yolk sac. The organisms are recovered from

purulent areas at the sites of the placental attachments. It is quite noteworthy that the guinea-pigs show no apparent clinical results of the injection.

Skin

Rosenow (1915b) has convincingly shown that erythema nodosum is caused by a diphtheroid. He isolated the causative organism from carious teeth, tonsils, blood, throat ulcers and the erythematous nodes of the patients, and was able to reproduce the specific lesions of this disease in dogs, rabbits and guinea-pigs. He used a large number of animals, and recovered the organisms from the reproduced lesions, as well as demonstrating them microscopically therein. He obtained the same results in eight clinical cases of this condition.

Dean, quoted by Graham-Smith (1908), recovered diphtheroid bacilli from a leprosy-like disease in rats, and found that the organism was mildly pathogenic for other rats.

Meninges and brain

Warnecke (1900) found diphtheroids in pure culture in a case of otitis media. Apparently from this condition meningitis and metastatic abscess developed, and from both situations the organism was recovered.

Heart

Howard, quoted by Hamilton (1904), has described an interesting case of ulcerative endocarditis without diphtheria. He recovered pure cultures of the organism from the heart valves, spleen and kidneys. The organism was non-pathogenic for guinea-pigs. In the light of the relation which certain members of this group bear to the streptococci, the significance of these findings becomes more pertinent.

Bones and joints

Rosenow (1914b) cites a case of a duodenal ulcer which developed a septic fever two weeks following operation. In

conjunction with systematically placed nodules along the subcutaneous vein of the forearm, the patient was afflicted with a very severe pain in the femur. Pus from a tonsil yielded a diphtheroid bacillus and a streptococcus. The blood yielded a diphtheroid bacillus in pure culture. A vaccine made from the latter organism caused a disappearance of all symptoms.

I have isolated a diphtheroid bacillus in pure culture from an acute swelling in both popliteal spaces in a case of chronic arthritis. A culture was obtained also from the blood. Agglutinin reactions were positive. Vaccines made from the organism cleared up the acute condition and very markedly relieved the chronic arthritis. Voigt[2] has isolated a diphtheroid in pure culture from the urethra in a case of gonorrhoeal rheumatism. This organism had much the same characteristics as my streptococcoid strain 1, and gave positive agglutination reactions with the patient's serum. An autogenous vaccine caused a marked improvement in the patient's condition, which was of significance inasmuch as he had failed to respond to a gonococcus vaccine. Experimentally the erythema nodosum diphtheroid and strain 1 of this series constantly produced arthritis.

LYMPHATIC SYSTEM

Bunting is the most prolific contributor to the diseases of this system. He has isolated a very pleomorphic, antiformin-fast, non-acid-fast, Gram-positive diphtheroid from the lymph glands of several cases of Hodgkin's disease. He considers this organism identical with the one described by Frankel and Much (1910) as the probable cause of this condition. He describes in detail the various stages of the tissue lesions which he has produced experimentally. They are typical of the pathology of this disease as described by Reed, Longcope and others. He has also produced in monkeys the blood picture which he considers characteristic of the condition in the human subject. Furthermore, this blood picture has been produced in man by the injection of the killed *B. Hodgkini*. He has also produced in mon-

[2] Personal communication.

keys the acute form of the disease which occasionally occurs in man. At the present time his experimental results remain unconfirmed.

Rosenow (1915a), Rhea and Falconer (1915), Kolmer, quoted by Fox (1915b), Klotz and Holman,[3] Torrey (1916), Fox (1915b), Wade and Harris (1915) and many others have isolated diphtheroid organisms from Hodgkin's lymph glands as well as from other locations, but they have never been able to show that they were pathogenic.

Bunting also claims to have isolated similar diphtheroids from Banti's disease and reproduced the condition in dogs. From pseudo-leukemia, lymphosarcoma and acute and chronic leukemia a diphtheroid similar to *B. Hodgkini* has been isolated by Bunting, who takes the position that these various conditions are different expressions of the toxic action resulting from various strains of the same diphtheroid subgroup.

Steele (1914) has described an antiformin-fast bacillus from the spleen in a case of acute leukemia which he thinks is identical with *B. Hodgkini.* Simon and Judd (1915) have confirmed Steele's observations. Their strain does not ferment glucose, lactose, sucrose, mannite or inulin. Steele's is negative on glucose or lactose, which seem to be the only sugars tested.

Hall and Stone (1916) have identified the bacillus of Preiz-Nocard in caseous lymphadenitis of sheep, and in the abscesses of eleven horses and sheep. It was found for the most part in pure culture, and they believe they have established it as the cause of the condition. It was pathogenic to most of the lower animals. The same organism has been demonstrated as the cause of ulcerative equine lymphangitis by Preiz-Nocard. The organism produces orchitis and generalized suppuration throughout the lymphatic system in guinea-pigs. It is a typical member of the *B. flavidus* subgroup.

I have studied Bunting's Banti and chronic leukemia strains, which are number 43 and 44 of my series. Although resembling his *B. Hodgkini* very closely morphologically and culturally,

[3] Personal communication.

they are totally opposed as far as the sugar reactions are concerned. Neither of them ferment any of the ten sugars tested, which compares with Simon and Judd's results. They resemble *B. Hoffmannii* very closely in this respect. Bunting's pseudoleukemia strain which I also have studied can be said to be practically identical with his *B. Hodgkini*, differing only in its fermentation of inulin and salicin (strain 45).

VIRULENCE

It is a very common occurence for diphtheroid bacilli to lose their virulence rather quickly after they have been removed from a favorable to an unfavorable environment. Davis (1898) and Williams (1898) to whose experiments I have referred already report that their organism lost its virulence very quickly, so that 11 cc. was the minimal lethal dose. When first isolated 2 cc. of the broth culture killed promptly in twenty-four hours. Hektoen's (1901) bacillus which produced hepatic cirrhosis in guinea pigs "lost its virulence very soon, so that the experiments could not be continued." Hamilton (1904) has had the same experience with several of her pathogenic strains.

My strain 2 was an example of this change. It was isolated as a strict anaerobe. I was able to get it to grow aerobically by using various oxygen pressures and gradually adapting it to an aerobic environment. When this had been accomplished it was no longer pathogenic for rabbits, even in very large doses. The anaerobic culture which had never been so changed, still retains its pathogenicity. This phenomenon naturally suggests the idea that the virulence may have been recently acquired.

SYMBIOSIS

The patient from whom I isolated strain 2 could trace his clinical condition directly to an untreated wound in the foot caused by a rusty nail. A continuous fever followed for six months at the end of which time he had a severe parenchymatous nephritis. This strain was isolated from the blood in pure culture, but in the urine it was mixed with a streptococcus.

It was not unlikely that the streptococcus and the diphtheroid were introduced at the same time. Dick (1915) in his series of nephritic cases finds the diphtheroid usually associated with a streptococcus. Hektoen's pathogenic diphtheroid was isolated from a case of blastomycotic dermatitis of the hand. Hamilton's pathogenic diphtheroids have been found chiefly in scarlet fever throats, and she suggests a symbiotic relationship to account for their pathogenicity. I have obtained some very suggestive experimental evidence along this line, but it is too incomplete to consider as yet. An apriori argument for such a contention lies in the wonderful lability of the group as a whole. Its ability to take on various forms I have already sufficiently emphasized. The same race may vary in its staining properties within wide limits. This I have noted in relation to Gram's stain, to acid-fast stains, and to the metachromatism with Loeffler's. Culturally it is one of the most adaptive of organisms, which may explain its characteristic viability. It is possible that a group with such a labile chemical base would be quite susceptible to symbiotic changes.

One of the most ideal places for such changes to be developed lies in the foci of infection in the crypts of the tonsils, pus pockets in the teeth, etc. It is Rosenow's idea that streptococci acquire their elective affinities in this way. It is noteworthy that many of his cases of erythema nodosum have yielded the infecting organism from one or both of these locations. Clinically this condition is a sequel of streptococcic tonsillitis, and a possible symbiosis is suggested. Bunting (1914) traces the source of infection of one of his Hodgkin's cases to an otitis media of some duration. Again, in a primary inguinal case the patient gave a history of a sharp attack of cystitis preceding glandular involvement. Infection through the gastrointestinal mucosa has been shown where Hodgkin's disease is confined to the lymphoid tissue of that tract and the mesenteric glands. In this connection, Hektoen (1901) had an interesting experience with his strain. The organism failed to produce the characteristic hepatic cirrhosis in rabbits that obtained in guinea pigs, unless it was introduced into the anterior chamber of the eye. Subcutaneous and intravenous doses did not affect the liver.

These facts lead very logically to the consideration of the validity of the guinea-pig test as usually carried out, viz., as an infallible criterion of virulence for the diphtheroid group. In addition to what already has been adduced, there is much more information that might be focused on this point. Cave, Priestly, Richmond and Salter, Prochaska, Hewlett and Knight, Fox and others have attributed to *B. Hoffmannii* a causal relationship to a benign unilateral tonsillitis with membrane formation. The organism is usually found in pure culture, and some of these authors do not hesitate to say that the failure of the guinea-pig to die is not conclusive negative evidence, when so much positive evidence points in the other direction. Hamilton (1904) shows that four strains of diphtheroids are non-pathogenic for man and yet are pathogenic for guinea-pigs. This illustrates converse evidence. De Witt (1912) proved that a diphtheroid was the cause of a septic fever in the human being, but notes that the organism was not pathogenic for guinea-pigs, especially after the first generation. In Howard's famous case (quoted by Hamilton) a diphtheria-like organism was isolated in pure culture from the heart valves, spleen, liver and blood, but it was non-pathogenic for guinea-pigs. Klein's (1903) organism was proven to have caused hepatization in the lung of white rats, yet was either non-pathogenic for guinea-pigs or caused only a local abscess.

The admonition of Rosenow in this regard can well be called to mind; this is to inject an animal, watch it carefully, kill it in a reasonable time and *look* for lesions. Teacher and Burton (1915) discovered an epidemic of infectious abortion among their guinea-pigs and showed that the strain on reinjection into a suitable animal produced placental separation without a detectable symptom on the part of the animal. Bunting was able to get a "take" only with one of his Hodgkin's strains by multiple injections into one monkey until local abscess was produced; by transferring the contents of this abscess directly to the second monkey, he was able to kill it with symptoms of sepsis.

DISCUSSION AND SUMMARY

It would seem that there is plenty of evidence for pathogenicity on the part of the diphtheroid group—at least it is not limited to a mere handful of cases. The virulent strains quickly lose this quality, which suggests that it has been of recent acquisition. Since this type of infection so often apparently has its origin in a focus, it would seem logical that the virulence may have been acquired in that location. This evidence, coupled with the fact that the group as a whole occupies a borderline position pathologically, leads to the idea of "sub-infection" introduced by Adami and others. Sub-infections are fundamentally conditional infections to a much greater degree than is the case with the ordinary virulent infection.

It would seem logical to consider an individual on the "border line" between health and disease before he becomes susceptible to a subinfection. In such a case the validity of the guinea-pig test as usually employed would not have its ordinary significance. One would not usually expect a healthy guinea-pig to be affected by an organism which only proved pathogenic for the human species when the resistance of the latter had been impaired. It would seem probable that our attempt to infect experimentally does not approximate human conditions closely enough, and that this is the reason why we fail. Our working margin for successful infection with diphtheroids might be compared to the rapid loss of "elective affinity" of Rosenow's streptococci on artificial cultivation. His work suggests that there are many low-grade infections in man, which insidiously give rise to a very definite pathology; these changes may, however, go unnoticed until the infection either culminates critically for the patient, or what is more important from our standpoint, prepares him for a more severe infection. The acute and therefore more noticeable character of the latter may cause us to ignore the insidious antecedent changes (sub-infections) which made the acute attack possible.

The demonstration of a biological link between the diphtheroid and streptococcic groups would seem to entitle the former

to more consideration from the standpoint of pathogenicity than it has received in the past. It suggests a plea for a better interpretation of these organisms. Their discovery in certain locations may have the same meaning as the discovery of streptococci of moderate virulence. A concrete instance will illustrate my point of view.

Some time ago I had occasion to study a case of severe arthritis which had a very plain gonorrhoeal history, and clinically was a case of this sort. The patient responded to a diagnostic dose of gonococci, but the vaccine had no curative effect.

Streptococcic vaccines improved the condition markedly. At this juncture the patient left the hospital to spend the week-end at a friend's home. She inadvertently spent too much time on her feet, and returned with a boggy swelling in each popliteal space about the size of a hen's egg. Puncture of each tumor under aseptic conditions yielded a pure culture of a very pleomorphic diphtheroid bacillus which fermented a wide range of sugars. Intravenous injections into rabbits developed, among other things, a non-suppurative arthritis which did not kill the animal. Subcutaneous injections in guinea-pigs caused only a transient edema at the site of injection. Autogenous vaccines gave beneficial results in the case.

I wish also to refer to the case of Voigt's (already cited), which he worked out in my laboratory. Strain 41 of this series came from the urethra of that case, and the sugar reactions will show that it belongs to the *B. enzymicus* sub-group.

The studies of Hine (1913) show that urethral diphtheroids characteristically have a wide range of fermentation. Ohlmacher years ago thought that he had beneficial results from incorporating them in his vaccines for chronic gonorrhoea. Strains 13 and 14 of my series came from a case of acute gonorrhoea, and strain 14 when given to young rabbits intravenously caused myositis, thrombosis, sepsis and death.

Further evidence bearing on this point may be derived by consulting the range of sugar fermentations of the diphtheroids isolated by Fox (1915b) from glands draining arthritic joints. Taken collectively they have a much wider fermentative range

than the other strains considered collectively. These collations are at least suggestive, and point out a direction for clinical research which may be of benefit.

Since Bunting's and my strain of *B. Hodgkini* belong to the same group and gave cross complement-fixations with the strain "Lewis streptococcus," more serious consideration of them may be merited. Lymph-gland studies show that a great diversity of diphtheroids inhabit these structures. For this reason much confusion has arisen regarding the identity of *B. Hodgkini.* Curiously enough there has been no adequate description of this organism. The sugar reactions which go a very long way toward defining it, have been omitted from nearly all descriptions. I have shown that strains 37, 38, 39, and 40 of Bunting's give uniform sugar reactions.

I have studied some of the Rosenow-Gaarde cultures, and most of them yielded at least two types of diphtheroids when plated out. The predominating type was the saprophytic *B. Hoagii* or some of its variants. Usually a member of the *B. enzymicus* group could be separated also, but this was not always the case. This experience corresponds to that of Torrey, Bloomfield, Fox and others who have studied the glandular diphtheroids. This fact may help to account for the negative animal experiments reported.

Four of these "Rosenow-Gaarde" cultures were kindly sent me by Dr. J. J. Moore from Dr. D. J. Davis's laboratory. Inasmuch as lymph gland cultures yield mixed types of this organism it would seem necessary to identify *B. Hodgkini* in such mixtures. This point is interesting in connection with Moore's recent paper on "Immunologic Studies in Hodgkin's Disease" (1916). He prepares a polyvalent antiserum with 16 strains of diphtheroids isolated by Drs. Rosenow and Gaarde from Hodgkin's glands. Although this serum fixes complement with the homologous antigen in doses of 0.0005 cc., there is no fixation with the sera of ten individuals having Hodgkin's disease. If a generous portion of the organisms used in this work did not belong to the same sub-group as those with which Bunting claims to have gotten his results, the work of the two observers would be difficult to compare.

The fact that little or no serological evidence has been brought forward is an argument against the current etiological theory. Torrey makes a discouraging observation regarding any positive evidence, inasmuch as he obtains an anaerobe (*B. lymphophilis*) from Hodgkin's glands which he says reacted positively to lymphosarcoma and Hodgkin's disease to which it bore no evident relation! The great variation in colony formation possessed by a single strain gives this characteristic little differential value. The pleomorphism on which so much stress has been laid must always be correlated with the more differential biologic features.

CONCLUSIONS

1. It would appear that the sum total of evidence shows that the diphtheroid group is one of great diversity and lability, and that it is more or less related to several other groups of organisms. Prominent among these is the acid-fast group, represented by *B. tuberculosis;* the streptococcus group, represented by some of its non-hemolytic types; *B. proteus* and the enterococcus. Symbiotic relations between these groups and the diphtheroids is more than a possibility, although as yet we have little definite knowledge on this phase of the subject.

2. The group may be divided into at least seven sub-groups, viz.; *B. Hoagii, B. diphtheroides liquefaciens, B. xerosis, B. Hoffmannii, B. enzymicus, B. flavidus* and *B. Ruedigeri*. Among the first four are found the greater number of saprophites, while the last three subgroups yield the larger number of examples of pathogenicity. *However, any of the subgroups may become pathogenic under favorable conditions.*

3. The sugar reactions form a fairly reliable criterion for the delimitation of these groups, when a sufficient number of sugars are employed and the various technical and other limitations regarding their utility in this respect are observed. The cultural, morphological and immunological characteristics can be successfully correlated with a grouping made on this basis. The most decisive group-differentiation resulted from the use of the complement-fixation reaction.

4. The recognition and delimitation of a subgroup related to the streptococci, *B. enzymicus*, may lead to a more serious regard for the pathogenicity of certain members of the group, and at least should demand a more careful study and interpretation of diphtheroids; *B. enzymicus* may be found anywhere in the body, especially in the blood, joints and upper respiratory tract.

5. The guinea-pig test as usually employed for the determination of the virulence of the Klebs-Loeffler bacillus is by no means infallible when similarly employed with the diphtheria-like bacilli. Rabbits may prove to be better test animals, as they are more susceptible, especially with the intravenous method of injection. However, when this class of organisms is not pathogenic for laboratory animals, the inference is not to be conclusively drawn that they are lacking in pathogenic importance for man. The conditions determining their invasion of the latter species can only be imperfectly paralleled when healthy representatives of another species are employed.

It gives me great pleasure to express my gratitude, first to Dr. M. J. Rosenau for his sympathetic and hearty coöperation in this study, and second, to Dr. J. P. Bill, Dr. J. H. Brown, Dr. W. G. Smillie and Dr. C. S. Spruit for innumerable miscellaneous assistances rendered. For transplants of interesting strains of this group I am obliged to Dr. F. B. Mallory, Dr. S. B. Wolbach, Dr. Wm. Hinton, and Dr. W. H. Watters of Boston; to Dr. Oscar Klotz of Pittsburgh, Dr. Herbert Fox of Philadelphia, Dr. C. S. Bunting of Madison, Wis., Dr. John Teacher of Edinburgh, Scotland, Dr. W. C. R. Voigt of Ann Arbor, Mich., and to Dr. D. J. Davis of Chicago.

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Professor H. W. Conn

PROFESSOR H. W. CONN

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It is a pleasure to write of the life and work of a teacher with whom the writer was so pleasantly associated in both teaching and investigation for sixteen years. Professor Conn takes his place among the prominent biologists of the nineteenth century. His achievements are particularly interesting and noteworthy because they cover the pioneer period of the branches of science which he made his specialties. He was at the front in the great advance made in biological science in the past thirty years, but at no time lost his deep interest in the social welfare of all mankind. His dominant work was in dairy bacteriology, although he taught and expressed some of the most profound principles of agricultural science in the relation of bacteria to crop production. He was always on the right side of all public and social problems, although he was given ample opportunities to testify on the questionable side of public welfare controversies at large remuneration.

As a teacher, perhaps, he reached his highest achievement, as is attested to by more than two thousand students who came under his influence. His personal magnetism, polished English, interesting illustrations and illuminating suggestions captivated his listeners. His teaching was masterful, and with him it became an art. He was able to make a difficult subject easily understood, and the breadth of his conception of the subject at hand was remarkable. This breadth of view acquired from very extensive reading made a lasting impression on the mind of the student. No one could attend his lectures without acquiring a wider and truer view of life and its problems. In laboratory teaching his pleasant suggestiveness and persuasive leading stimulated the student to acquire knowledge for the mere pleas-

sure of it. His was the method of Agassiz. He instructed his assistants to give as little help as possible until the student had reached the limit of his own observation. "Let them find it out for themselves," was the trite saying which proved that he well knew the true methods of acquiring an education. He was able by clear and concise directions to conduct laboratory sections in general biology for over seventy students at a time.

As a member of society he could converse entertainingly and instructively on almost any topic of interest. He had the courage to attack any problem, no matter how difficult, and always labored at its solution with great perseverance.

The most interesting phase of his life's work was accomplished in dairy bacteriology. At the time of the founding of the Storrs Agricultural Experiment Station by Dr. Atwater in 1889, Dr. Conn commenced the studies of the organisms which ferment milk. In the first report of this Station will be found an account of this early work. In 1890 he isolated bacterial enzymes from pure culture which when added to milk in powder form caused a curdling and subsequent digestion. In the fall of 1891 was commenced the study of the organisms that ferment ripening cream. As each kind of bacteria was isolated it was given a number. When the exhibit was installed at the World's Fair, Chicago, in 1893, to show the effect of bacteria in the production of flavor in butter, some thirty-five varieties of organisms had been isolated. No. 2 at that time produced the best flavor, and No. 16 the worst. The latter was sickening, brackish, and persistent. These two organisms were used daily at the fair in the ripening of cream for churning. An upright case was installed in the exhibit. This case held about forty large test tubes which showed the effect of pure cultures growing in milk. The exhibit attracted a great deal of attention and the city papers published long descriptions of it. During the same summer eight or nine varieties of bacteria were added at the fair to the list, most of them isolated from a can of milk from Uruguay. Among them was the remarkable and famous "B 41." Following this came the development and application of cream starters which gained practically universal use throughout the

United States. At the same time blue litmus gelatin was developed for the isolation of acid bacteria. From this experimental work in the bacteriology of milk has been built up a fairly extensive science of dairy bacteriology.

As director for several years in the Summer School at Cold Spring Harbor, Long Island, his capabilities reached their highest achievement. The writer's memories of him at this place are the most cherished of all. We were a happy family. All restraint of forced attendance was removed, but we could not afford to miss a single exercise. His lectures here, as elsewhere, were models of presentation and scope. As there was no church in the immediate vicinity, Dr. Conn would address us Sundays on some appropriate topic.

In his interest of scientific agriculture for the welfare of mankind, and with keen perception and prophetic vision, he presented the problems many years ago which we are today trying to solve. In 1882 Atwater and Woods demonstrated that the legumes were capable of fixing definite amounts of atmospheric nitrogen. Nine years later Professor Conn explained the function of bacteria in the fixing of this nitrogen in the root nodules of the legumes. To the writer a solution of these problems for the benefit of the world appears to be a most worthy goal.

Dr. Conn's influence as a Christian gentleman who was always an exponent of right, teaching the profound philosophy of correct living, will long make itself felt among his many students, associates and friends.

THE FAMILIES AND GENERA OF THE BACTERIA

PRELIMINARY REPORT OF THE COMMITTEE OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS ON CHARACTERIZATION AND CLASSIFICATION OF BACTERIAL TYPES

C.-E. A. WINSLOW, *Chairman*, JEAN BROADHURST, R. E. BUCHANAN, CHARLES KRUMWIEDE, JR., L. A. ROGERS, AND GEORGE H. SMITH

In the last presidential address before the Botanical Society of America, Dr. A. S. Hitchcock presented an interesting comparison between the dominant position of taxonomy in the botany of the eighteenth and early nineteenth centuries and its relative neglect today, and made a strong plea for the recognition of the very real importance of this branch of botanical science. "To me," he says, "the two great questions that botanists seek to answer are, "*How do plants live?*" and "*How are plants related?*" Most botanical investigations can be used as an aid in answering one or the other of these questions. From this standpoint the two fundamental divisions of botany are physiology and taxonomy." (Hitchcock, 1916.)

Botanists engaged in the study of the higher plants may perhaps neglect systematic problems with a certain degree of impunity, because so many of the more fundamental questions involved have been settled by the workers of an earlier period. Bacteriology however has never passed through a taxonomic phase; and it is unnecessary to rehearse at length our sound reasons for dissatisfaction with the present crude state of bacterial classification. It is obvious to all students of this group that the characterization of specific types is generally obscure and that the classification of these types into larger generic and family groups is almost ludicrously artificial.

With the first of these problems, the characterization of bacterial species, we are not at present concerned. The Society of American Bacteriologists at its Urbana meeting specifically

directed its Committee on Bacterial Classification to prepare an outline of classification of the Bacteria into families and genera. It is this task which we have attempted, with full recognition of the impossibility of reaching a final result, but in the belief that our present state of knowledge makes possible material advances over the classifications now in common use. We have been all the more ready to undertake the work because we have felt that if it were not undertaken by bacteriologists it would probably be attempted by botanists, unfamiliar with the peculiar characteristics of the organisms concerned.

I. PREVIOUS SYSTEMS OF BACTERIAL CLASSIFICATION

The present chaotic condition of bacterial classification is in large measure due to the carrying over into bacteriology of the conception—valid enough among the higher plants—that classification must be based primarily on visible structure or morphology. It has been pointed out in a presidential address before the Society that physiological characters are quite as suitable for systematic uses as structural differences.

> There is no fundamental distinction between morphological and physiological properties, since all are at bottom due to chemical differences in germ plasm, whether they happen to manifest themselves in the size and arrangement of parts or in the ability to utilize a certain food stuff. Indeed biochemical properties have a peculiar and unique significance among the bacteria, since it is precisely along the lines of metabolism that these organisms have attained their most remarkable differentiation. The higher plants and animals have developed complex structural modifications to enable them to obtain food materials of certain limited kinds. On the other hand the bacteria have maintained themselves by acquiring the power of assimilating simple and abundant foods of varied sorts. Evolution has developed gross structure in one case without altering metabolism; it has produced a diverse metabolism in the other case, without altering gross structure. There is as wide a difference in metabolism between the pneumococci and the nitrifying bacteria as there is in structure between a liverwort and an oak. (Winslow, 1914.)

Yet the only bacterial genera named and described up to 1896, and the ones which are still in general use today are based solely on morphological or structural characters, some of them of no significance, whatever, while physiological differences of the most fundamental importance are ignored.

The foundation for the current classification of bacteria was laid by Ehrenberg (1838) nearly a century ago. He divided his Vibrionia, which corresponded to the Bacteria, into five genera: *Bacterium* (straight, rigid filaments); *Vibrio* (straight filaments, showing serpentine motion); *Spirillum* (rigid spiral filaments); *Spirochaeta* (flexible spiral filaments); and *Spirodiscus* (spirals flattened, disc-like). Hoffmann (1869) recognized that the spherical forms previously treated separately must be classified with the other bacteria under Hallier's name of *Micrococcus*. Ferdinand Cohn in 1872 laid special stress on the grouping of the bacterial cells in chains or in zooglea masses. He distinguished four main groups (Tribus): Sphaerobacteria, spherical or oval, non-motile cells, often in zooglea masses (*Micrococcus*); Microbacteria, short cylindrical cells, motile, often in zooglea masses, (*Bacterium*); Desmobacteria, growing in threads without zooglea (*Bacillus, Vibrio*); Spirobacteria, motile spirals, without zooglea (*Spirochaeta, Spirillum*). Three years later Cohn proposed another classification in which these and other genera of the bacteria were separated from each other and grouped respectively with supposedly similar forms belonging to the blue-green Algae.

Perhaps the most significant bacterial classification after that of Ehrenberg was the system proposed by Zopf (1883–5). He retained a distinct group for the spherical bacteria (Coccaceae), reunited the rods and the spirals (Bacteriaceae); and introduced the groups Leptothriceae and Cladothriceae for the filamentous forms. His genera are tabulated below. The most important advance in this classification was the differentiation between the spore-forming organisms (*Bacillus*) and the non-spore-formers (*Bacterium*).

ZOPF'S CLASSIFICATION

I. Coccaceae

(Spherical elements, no spores, division in one or several planes)

1. *Streptococcus.* (Chains)
2. *Merismopedia.* (Plates)
3. *Sarcina.* (Packets)
4. *Micrococcus.* (Irregular masses)
5. *Ascococcus.* (Similar to Micrococcus, but showing marked zooglea formation)

II. Bacteriaceae

(Cocci, straight or curved rods and straight or spiral filaments, latter never showing differentiation between base and apex, division in one plane)

1. *Bacterium.* (Cocci and rods without spores)
2. *Spirillum.* (Spirals without spores)
3. *Vibrio.* (Spirals with spores)
4. *Leuconostoc.* (Cocci and rods, former showing spore formation)
5. *Bacillus.* (Cocci and rods with spores)
6. *Clostridium.* (Like *Bacillus*, but spores in spindle-shaped elements)

III. Leptothriceae

(Cocci or rods, threads or spirals, threads showing differentiation between base and apex, no spores.)

1. *Crenothrix.* (Threads with sheath, cells without sulphur, water forms)
2. *Beggiatoa.* (Threads without sheath, cells with sulphur granules, water forms)
3. *Phragmidiothrix.* (Cells without sheath, segmentation pronounced, cells without sulphur, water forms)
4. *Leptothrix.* (Threads, with or without sheath, segmentation not pronounced, cells without sulphur)

IV. Cladothriceae

(Cocci, rods, threads or spirals, threads showing false branching. No spores)

1 *Cladothrix*

Van Tieghem and de Bary laid even more stress on spore formation, dividing the bacteria sharply into an endosporous and an arthrosporous series. Hueppe followed Zopf's classification quite closely, introducing the genera *Staphylococcus* and *Leuconostoc* among the Coccaceae, separating the spiral form under the group-heading Spirobacteriaceae, and rearranging the genera under this head as follows:

Spirobacteriaceae

1. *Spirochaeta.* (Without endospores)
2. *Spirillum.* (With endospores)
3. *Vibrio.* (With endospores, and change of form in spore formation)

The same general plan of classification, based on cell form and spore production, was carried a step further by Lehmann and Neumann in 1896. The following outline is from the 1904 edition of their work.

I. Coccaceae

1. *Streptococcus.* (Dividing in one plane)
2. *Sarcina.* (Dividing in three planes)
3. *Micrococcus.* (Irregular division, including all but clearly marked chains and packets)

II. Bacteriaceae

1. *Bacterium.* (Without endogenous spores, rods usually 0.8–1.0μ in diameter)
2. *Bacillus.* (With endogenous spores, rods often more than 1.0μ in diameter)

III. Spirillaceae

1. *Vibrio.* (Short, rigid, slightly curved cells, with 1 to 2 polar flagella)

2. *Spirillum.* (Long rigid spiral cells, with lophotrichic flagella)
3. *Spirochaeta.* (Long flexible spiral cells, flagella unknown, motility accomplished by an undulating membrane)

Supplementary Group

ACTINOMYCETES

Thread-like cells with true branching, sometimes even a richly branching mycelium. Young cultures often show only normal unbranched bacterial cells. Many forms show a tendency to the formation of irregular clubbed cells.

1. *Corynebacterium.* (Slender, often slightly curved rods, often with a tendency to club formation, branching rare in young cultures and often difficult to find even in old ones. Neither flagella nor spores known. With weak stains show barred staining. Not acid-fast)
2. *Mycobacterium.* (Same as above except for the fact that the cells are acid-fast and take ordinary stains with difficulty, and that club forms are very rare outside the body)
3. *Actinomyces.* (Long, mycelial threads, showing true branching. Spores formed by fragmentation and cross division of filaments. Many forms produce a mold-like aerial mycelium. Not acid-fast. Motility sometimes present. Most forms produce a moldy odor)

This outline of Lehmann and Neumann's seems to us the most satisfactory classification that could be worked out on purely morphological grounds. In 1894, however, Migula suggested a new system in which motilty was used instead of spore formation as a secondary basis of classification. The outline below is from his later work, System der Bakterien (1900).

MIGULA'S CLASSIFICATION

Order *Eubacteria*

Cells without nuclei, sulphur or bacteriopurpurin, colorless or but slightly colored, some forms chlorophyll-green.

Family I. COCCACEAE

Cells when free spherical, in division sometimes elliptical. Division in one, two or three planes of space without previous elongation of the cell. Motility and endospore formation rare.

1. *Streptococcus*. (Spherical cells dividing in one plane, often remaining attached to each other to form pairs or chains. Zooglea-like sheath or capsule common. Non-motile. No endospores)
2. *Micrococcus*. (Division in two planes, sometimes forming bands of cells. Non-motile, endospores probably absent)
3. *Sarcina*. (Division in three planes at right angles to each other, forming packets when the cells remain attached to each other. Non-motile. Endospore formation doubtful)
4. *Planococcus*. (Division in two planes. Flagella present, usually 1 to 2 in number. No endospores known)
5. *Planosarcina*. (Division in three planes, pairs and tetrads usually seen rather than packets. Flagella present, usually one to each cell. No endospores)

Family II. BACTERIACEAE

Cells cylindrical rods in free condition, dividing in a plane at right angles to their length. Short-celled forms may be distinguished from cocci by elongation before division. Cells may remain united forming long or short threads. No sheath.

1. *Bacterium*. (No flagella. Some form spores, others do not)

2. *Bacillus*. (Peritrichic flagella. Spore formation common)
3. *Pseudomonas*. (Polar flagella, 1 to 10 in number. Endospores in some forms)

Family III. Spirillaceae

Cells more or less spirally curved, division in one plane at right angles to long axis. Spore formation rare. Most forms motile, with polar flagella.

1. *Spirosoma*. (Broad rigid cells, non-motile, free or in small zooglea groups)
2. *Microspira*. (Cells usually comma- or sausage-shaped, motile with one or rarely 2 to 3 polar flagella. Endospores not observed)
3. *Spirillum*. (Long or short spirals, lophotrichic flagella, spores sometimes observed)
4. *Spirochaeta*. (Slender spiral cells, generally long and flexible, showing serpentine and screw-like motions. Organs of motility unknown. Spores not observed)

Family IV. Chlamydobacteriaceae

Cylindrical cells arranged in threads with sheath. Reproduction by conidia which arise directly from the vegetative cells and develop into new threads without any resting stage.

1. *Chlamydothrix*. (Unbranched filaments, segments often demonstrable by the use of reagents. Connidia non-motile)
2. *Crenothrix*. (Unbranched filaments with differentiation between base and apex. Sheath thick and in iron waters often permeated with hydrate of iron. Cells cylindric or flat discoidal, conidia non-motile)
3. *Phragmidiothrix*. (Very long threads with delicate sheath. Conidia non-motile)
4. *Sphaerotilus*. (Cells in dichotomously branched threads, conidia motile)

Order *Thiobacteria*

Cells with no nucleus but with inclusions of sulphur, colorless or colored red, rose or violet with bacteriopurpurin. Never green.

This order includes the families Beggiatoaceae (genera *Thiothrix* and *Beggiatoa*) Rhodobacteriaceae (genera *Thiocystis, Thiocapsa, Thiosarcina, Lamproystis, Thiopedia, Amoebobacter, Thiothece, Thiodictyon, Thiopolycoccus chromatium, Rhabdochromatium* and *Thiospirillum*) into the classification of which we need not enter here.

An even more elaborate morphological classification of the bacteria is that suggested by A. Fischer in 1895, and later elaborated and altered in successive editions of his Vorlesungen über Bakterien. The outline below is taken from Coppen Jones' translation of the latter work (Fischer, 1900).

Order I. *Haplobacterinae*

Vegetative phase unicellular, spherical, cylindrical, or spirally twisted; isolated or united in chains or clusters.

Family I. Coccaceae

Vegetative cell spherical.

Sub-family I. *Allococcaceae*

Planes of fission without definite sequence; no pronounced colonies or growth-forms, cells isolated or in short chains or irregular clusters.

Genus *Micrococcus* Cohn. Non-motile. Includes most cocci, the pathological 'staphylococci,' etc.

Genus *Planococcus* Migula. Motile.

Sub-family 2. *Homococcaceae*

Planes of fission in definite sequence.

Genus *Sarcina* Goodsir. Three planes of division at right angles to each other. Cubical colonies, non-motile.

Genus *Planosarcina* Migula. Similar to *Sarcina*, but monotrichous, ciliate, and motile.

Genus *Pediococcus* Lindner. Two planes of fission, alternate and at right angles. *Micrococcus tetragenus Thiopedia* (a sulphur organism), and probably some species usually termed Micrococcus.

Genus *Streptococcus* Billroth. Planes of fission parallel, giving rise to chains; the pathological *Streptococci* and *Leuconostoc.*

Family 2. BACILLACEAE

Vegetative cell straight, cylindrical, ellipsoidal or egg-shaped; very short forms difficult to distinguish from cocci. Fission always transverse.

Sub-family I. *Bacilleae*

Spore-forming rods cylindrical, unchanged.

Genus *Bacillus* Cohn. Non-motile. *B. anthracis*, *B. diphtheriae*, etc.

Genus *Bactrinium* A. Fischer. Motile, monotrichous, with terminal cilium: includes provisionally all monotrichous rods whose spores are as yet unknown, e.g., *B. pyocyaneus.*

Genus *Bactrillum* A. Fischer. Motile, with lophotrichous ciliation. Includes provisionally *B. cyanogenus*, and many other sporeless forms.

Genus *Bactridium* A. Fischer. Motile, peritrichous, in some spores as yet unknown. Very numerous representatives, e.g. *B. subtilis*, *B. megatherium*, *B. vulgaris* (old genus Proteus), *B. typhi*, and *B. coli.*

Sub-family 2. *Clostridieae*

Rods spindle-shaped during sporulation.

Genus *Clostridium* Prazmowski. Motile, peritrichous; includes some of the butyric bacteria. Genera with monotrichous and lophotrichous ciliation are unknown as yet.

Sub-family 3. *Plectridieae*

Rods drumstick-shaped during sporulation.

Genus *Plectridium*, A. Fischer. Motile, peritrichous; some butyric bacteria, the organism causing tetanus, and a methane fermenter.

Family 3. SPIRILLACEAE

Vegetative cell cylindrical, but spirally twisted. Fission always transverse.

Genus *Vibrio* Miller and Löffler. Very slightly curved rods, 'comma'-shaped; motile, monotrichous. *Vibrio cholerae asiaticae* and numerous other vibrios of fresh and salt water.

Genus *Spirillum* Ehrenberg. Cylindrical cells twisted in an open spiral; motile, lophotrichous. *Spirillum undula*, *Sp. rubrum*.

Genus *Spirochaeta* Ehrenberg. Cells long and attenuated, spirally twisted with numerous turns; cilia unknown; the cell membrane is perhaps yielding. *Spirochaeta Obermeieri* (remittent fever).

Order 2. *Trichobacterinae*

Vegetative phase an unbranched or branched filament or chain of cells, the individual members of which break off as swarm-spores (*gonidia*).

Family I. TRICHOBACTERIACEAE

(a) Filaments non-motile, rigid, enclosed in a sheath.

Genus *Crenothrix* Cohn. Filaments unbranched and devoid of sulphur granules.

Genus *Thiothrix* Winogradsky. The same, but containing sulphur granules.

Genus *Cladothrix* Cohn. Filaments branched, false dichotomy (includes *Sphaerotilus*).

(b) Filaments motile, with oscillating and gliding movements, and devoid of a sheath.

Genus *Beggiatoa* Trevisan. Containing sulphur.

The prominent place given to motility seems to us to constitute a peculiar infelicity in these schemes. Spore formation marks off at least two fairly distinct natural groups of bacteria; but motility is a character of singularly slight taxonomic importance among the bacteria.

No one familiar with the colon group can hold that it is reasonable to place the common type of motile colon bacillus in the genus *Bacillus* along with *B. mycoides*, *B. aerogenes*, *B. anthracis*, *B. prodigiosus*, *B. radicicola* and *B. tetani*, and to place an organism having all its other properties identical but lacking flagella in the genus *Bacterium*. The same arguments hold true against the genera *Planococcus* and *Planosarcina* among the cocci. We find in several of the major groups motile and non-motile forms which are precisely alike in half a dozen respects and are clearly minor varieties of the same type, and it is absurd to give them generic rank, and group together widely different types which are alike in no single respect except that they have flagella. These genera based on motility are on a par with a division of animals into those with wings and those without, which would place bats and birds and flying fishes and bees in one group, and cats and ordinary fishes and worker ants in another. (Winslow, 1914.)

Unfortunately, however, it is precisely Migula's defective classification which has for some reason established itself in the literature of American bacteriology. Chester (1901) accepted it *in toto*, with the addition of Lehmann and Neumann's family *Actinomycetes*, which he called *Mycobacteriaceae*, and in which he included two genera only, *Mycobacterium* and *Streptothrix*. Erwin Smith (1905) also adopted the view that genera should be fixed by morphological characters only and accepted Migula's genera with the change of three names. He substituted *Vibrio* Miller for *Microspira* Schroeter; *Bacterium* Cohn for *Pseudomonas* Migula; and Aplanobacter (nov. gen.) for *Bacterium* Ehrenberg.

The classifications of Zopf and Lehmann and Neumann seem to us to be very much more satisfactory than those of Migula and Fischer. Like all the earlier workers in bacteriology, however, these authors left the great mass of the rod-shaped bacteria, including hundreds of enormously varied types, in the two gen-

era, *Bacterium* (small non-spore-formers) and *Bacillus* (large spore-formers).

It is to W. Kruse (1896) that we owe the first recognition that these unwieldy and heterogeneous genera must be broken up by taking into account the physiological as well as the morphological characters of the organisms concerned.

Kruse enumerates fifteen different sorts of characters by which the bacteria may be classified, giving first place to morphological differences, such as the spore formation, the presence of a gelatinous envelope, and motility, but recognizing also the importance of staining reactions, ability to develop in certain culture media, relation to temperature and oxygen, biochemical powers, pigment production, pathogenicity and character of growth in media. It is true that he considers the physiological characters as of minor and doubtful value and so variable as not to warrant giving the groups of bacteria definite generic names. Yet he presents at the end of his discussion the following list of numbered groups which represents by far the most valuable plan of classification suggested up to 1909. There will be little question of the fact that most of the types listed represent natural groups easily recognizable by all working bacteriologists. In the characterizations below Kruse's summary table (pp. 93–95) has been in some cases supplemented by data given in his text.

KRUSE'S CLASSIFICATION

I. Coccaceae

A. *Streptococcus*. Division in one plane
   1. Saprophytic streptococci, usually short, often liquefying gelatin, sometimes showing marked zooglea formation (*Leuconostoc*).
   2. Parasitic streptococci, usually long
      a. *Diplococcus pneumoniae* type
      b. *Streptococcus pyogenes* type

B. *Merista*. Division in two planes at right angles to each other.
   1. Tetragenic group. Typical arrangement of cells in tetrads

2. *B. gonorrhoeae* group. Cells usually in pairs. Gram-negative
3. *Staphylococcus pyogenes* group. Development in two planes often interrupted, giving pairs, tetrads and also short chains

C. *Sarcina.* Development in three planes at right angles to each other.

II. Bacillaceae

1. Group of the colorless sulphur bacteria; (*Beggiatoa, Thiothrix*) usually large unbranched threads, without spores, utilizing sulphur in their metabolism
2. *Leptothrix* group. Non-cultivable water organisms, sulphur-free, unbranched, which form large threads without spores
3. *Cladothrix* group. Usually non-cultivable water organisms which form large threads and show false branching. (Spores formed in *Cladothrix intricata* which can also be cultivated in nutrient media)
4. Hay bacillus group. Usually large bacilli, commonly isolated or in small groups, when in long chains never showing base and apex differentiation as in many forms of the first three groups. Spores formed without change in the shape of the mother cell. Gram-positive. Saprophytes. Easily cultivable. Generally liquefy gelatin. Spore germination probably equatorial
5. Anthrax group. Distinguished from preceding group only by fact that spore germination is probably polar
6. Malignant Oedema group. Large spore-bearing anaerobic bacilli. Saprophytic or parasitic. Colonies on agar usually stellate. No change in form of mother cell on spore formation. Less easily stained by Gram method than preceding group. Usually liquefy gelatin and produce foul odors

7. Symptomatic Anthrax and Butyric Acid group. Differs from preceding group in forming clostridium spores
8. Tetanus group. Differs from preceding groups in forming drumstick spores
9. Proteus group. Aerobes or facultative anaerobes of medium size decolorized or irregularly stained by Gram. No spores. Colonies resemble those of preceding groups in showing amoeboid or stellate outgrowths sometimes separating as daughter colonies; also in producing malodorous decomposition of protein. Cells vary from coccoid forms to long, sometimes twisted filaments

   Supplementary group: liquefying, pathogenic forms
10. Fluorescent group. Non-spore-bearing bacilli, generally of medium size. Gram-negative
11. Pigment-forming group. A somewhat artificial group including red, brown, yellow, blue, and violet chromogens
12. Water Bacilli. Easily cultivable saprophytic bacilli of medium size, non-spore-forming, Gram-negative, usually motile and gelatin-liquefying. (Root nodule bacteria and plant pathogens included)

    Supplementary group: marine phosphorescent forms, often curved rods
13. Nitrobacteria. *Nitrosomonas* and *Nitrobacter* types. (Nitrogen fixers of the soil also included)
14. Aerogenes and Rhinoscleroma group. Non-motile, non-spore-bearing, non-liquefying, Gram-negative bacilli of medium size, usually plump. Easily cultivable. Facultative anaerobes. Tendency to capsule formation. Surface colonies on gelatin drop-like

    Supplementary group: Acetic and lactic acid bacteria, and bacteria of cheese and slimy fermentation

15. Colon-Typhoid group. Distinguished from preceding group by motility, and with less constancy by lack of capsules, more expanded surface colonies on gelatin and tendency for rods to be longer and more slender
16. Haemorrhagic Septicemia group. Distinguished from preceding group by high pathogenicity. Motile or non-motile
    Supplementary group. Bacilli of human haemorrhagic infection (includes *B. pestis*)
17. B. tenuis sputigenus group. Gram-positive, non-spore-bearing, non-liquefying, bacilli of varying size. Motile or non-motile. Facultative anaerobes
18. Influenza group. Very small isolated bacilli, Gram-negative, non-spore-bearing, difficult of cultivation, most forms obligate parasites
19. *Swine erysipelas* group. Differing from preceding group by Gram-positive staining. Moderate growth on media. Highly pathogenic
20. Glanders and Pseudotuberculosis group. Small bacilli isolated or in chains, non-spore-forming, usually Gram-negative, growing poorly on media. Specially characterized by slow metastatic growth in tissues and production of granulating centers
21. Diphtheria group. Small or medium-sized bacilli, swollen and clubbed in older cultures. Non-spore-bearing, Gram-positive, non-liquefying. Obligate parasites. Pathogenic forms either develop locally producing a powerful toxin or produce metastases as in preceding group. Dividing cells sometimes slip round into parallel position forming palisade grouping. Barred and granular staining in old cultures. Branched cells occasionally observed
22. Tuberculosis group. Small, slender, non-motile bacilli, Gram-positive, highly resistant to ordinary stains. Probably non-spore-forming, obligate parasites, growing very slowly on media. Metastatic

development in body. Tendency to irregular staining. Club forms and branched forms occur

III. Spirillaceae

1. Saprophytes, cultivated with difficulty or not at all
2. Saprophytes, cultivable, non-liquefying
3. Saprophytes and parasites, easily cultivable, liquefying
4. Obligate parasites

Kruse's treatment of the spherical and spiral forms is obviously very casual (as he admits in a footnote). His groups of the bacilli however show wide knowledge and clear analysis. With our present information we can combine some of his groups and subdivide others; but most of them represent clear and definite entities, and the outline above deserves the serious consideration of anyone engaged in the task of systematic bacteriology.

The next contribution to the broader questions of taxonomy was the work of the Winslows (1908) on the spherical bacteria. They emphasized two points in particular, the value of the frequency curve in fixing specific types and the importance of correlations of characters in defining generic groups.

The application of the second principle led these authors in the first place to recognize two distinct subfamilies within the *Coccaceae*. The cocci of the first group, comprising most of the forms from the body, showed as a rule chains and irregular cell-grouping, stained by the Gram method, gave a meager or only fair growth on media, formed acid from carbohydrates and produced no pigment or a white or orange one. The cocci of the other group, for the most part from soil or water, often showed packet formation, were usually Gram-negative, grew well on media, failed to ferment carbohydrates and produced a yellow or red pigment. "Each character was occasionally found in the group where it did not usually occur; but the correlation of properties in the vast majority of cases was very strong."

The definitions of subfamilies and genera were given as below:

WINSLOWS' CLASSIFICATION

Coccaceae

A. Subfamily *Paracoccaceae*

Parasites. Growth not abundant (or, one species, zooglea-forming saprophytes, yielding abundant growth in saccharose media). Generally Gram-positive. Acid formers.

1. *Diplococcus*. Cells in capsulated pairs. Parasites. Growth very meager. Inulin fermented. No pigment
2. *Ascococcus*. Cells in chains occurring in masses of zooglea in sugar refineries. Aberrant saprophytic form. Growth abundant in saccharose media. No pigment
3. *Streptococcus*. Cells in chains. Parasites. Growth meager. Inulin not fermented. No pigment
4. *Aurococcus*. Cells in irregular groups. Parasite. Growth fair. Orange pigment
5. *Albococcus*. Cells in irregular groups or in fours. Parasites. Growth good. White pigment

B. Sub-family *Metacoccaceae*

Saprophytes. Growth abundant. No zooglea. Generally Gram-negative. Not acid formers.

1. *Micrococcus*. Cells in irregular groups. Pigment generally yellow
2. *Sarcina*. Cells in packets. Pigment yellow
3. *Rhodococcus*. Cells in irregular groups or packets. Pigment red

It appeared from this study that the packet grouping to which so much importance had previously been attributed was correlated with no other significant character, the genera *Micrococcus* and *Sarcina* being absolutely parallel to each other in all other respects. On the other hand, pigment formation which had been so lightly thought of that the orange, yellow and white staphylococci were treated as varieties of a single species, was markedly correlated with important physiological differences.

Thus the white and orange chromogens were usually parasitic in origin, Gram-negative, and non-fermenters, while the yellow and red forms differed in all these respects. Even the closely related white and orange groups, while both included liquefiers and non-liquefiers, showed a perfectly characteristic difference in the amount of liquefaction produced by the liquefying forms, the white cocci liquefying slowly and the orange cocci very rapidly. The red cocci showed feeble liquefaction and vigorous nitrate reduction.

Buchanan (1915) has justly questioned the validity of the generic terms applied to their groups by the Winslows, and has pointed out that many of the names should be modified to conform to the International Rules for Botanical Nomenclature.

There remains to be considered but one other system of bacterial classification, the revolutionary and illuminating monograph of Orla Jensen (1909).

The chief contention of this author concerns the supreme importance of physiological characters, which he maintains should furnish a primary basis of classification among the bacteria just as chemical composition is more fundamental than crystalline form in the classification of minerals. He points out the significant fact that the three major morphological types of the ordinary bacteria are paralleled in the clearly homogeneous group of the red sulphur bacteria.

The presence or absence of flagella Jensen rightly considers as of very minor importance. The arrangement of the flagella, when they are present, happens on the other hand to be correlated with fundamental physiological differences, and the author gives the two orders into which he divides the bacteria the names of *Cephalotrichinae* (monotrichous or lophotrichous) and Peritrichinae (peritrichous). The *Cephalotrichinae*, deriving their life energy almost entirely from oxidative processes, are all water or moist earth forms, with the exception of a few peculiar plant and animal parasites, and for the most part grow badly or not at all on ordinary organic media, and spores are never formed. The second order, the *Peritrichinae*, includes the more specialized bacteria in whose metabolism the splitting of carbohydrates or amino-acids plays a primary rôle rather

than oxidation or denitrification. They are rods or cocci, peritrichous when possessing flagella at all, and among them are found all the commoner putrefactive and parasitic types.

In general the bacteria which derive their life energy from inorganic sources are clearly most primitive, while Jensen considers those forms which derive their energy from carbohydrates and their nitrogen from inorganic nitrogen compounds as coming next, the types requiring nitrogen in organic form being the highest. He points out that the opportunity to utilize such substances as milk sugar and urea must necessarily have come only in relatively recent geologic time, and that human pathogens must be among the youngest of bacterial types.

Resting in the main on a detailed consideration of those metabolic processes, Jensen divides the *Cephalotrichinae* into seven and the *Peritrichinae* into four families. The first series begins with the Oxydobacteriaceae, including the most primitive bacteria, which oxidize methane and carbon monoxid, the nitrifiers, the acetic acid bacteria and the Azotobacter group. Then follows the Actinomyces family which includes the root nodule bacteria and the mycobacterium (tuberculosis) group. The collocation of the latter forms is startling at first, but their morphology, their oxygen requirements and their unique pathological relations, almost symbiotic by contrast with the quick toxic action of other pathogenic bacteria, offer some evidence of real relationship. The third, fourth and fifth families are the Thiobacteriaceae (the sulphur bacteria), the Rhodobacteriaceae (the red or purple sulphur bacteria) and the Trichobacteriaceae (*Cladothrix*, *Crenothrix*, *Beggiatoa*, etc.) which are clearly natural groups. The last two families, the Luminibacteriaceae and the Reducibacteriaceae, are typically denitrifying organisms which form a connecting link between the primitive oxidizing bacteria and the *Peritrichinae*. They include the fluorescent water bacteria and the phosphorescent vibrios and at the higher end of the series such types as the cholera organism in which the ability to split complex products with the formation of lactic acid and indol begins to appear.

The second order, the *Peritrichinae*, is divided into four fami-

lies. The first, the Acidobacteriaceae, includes the non-spore-forming carbohydrate fermenting types, among the principal representatives being the colon-typhoid group and practically all the cocci. His second family, the Alkalibacteriaceae, shows a higher development of the power of decomposing nitrogenous bodies, and includes the liquefying proteus forms, the actively liquefying aerobic spore formers and certain urea fermenters. The last two families, the Butyribacteriaceae and the Putribacteriaceae, are made up of the strict anaerobes.

The broad outlines of this classification are highly suggestive, and the emphasis on biochemical properties is a long step in the right direction. When one studies the families and genera in detail, however, the result is somewhat disappointing. Professor Jensen rides his physiological principle much harder than even Migula did his morphological one, and makes no attempt at a judicial consideration of the grouping of common characters which should mark natural biological subdivisions. His disregard of the rudimentary principles of terminology is absolute. He coins new names for well established groups with entire freedom, according to the simple principle that the names of rod forms with polar flagella should end in *monas*, or rod forms with peritrichic flagella in *bacterium*, or spore-bearing rods in *bacillus*, etc., and with these suffixes he compounds roots expressing the chemical activity of the organisms concerned. Thus for example he changes *Azotobacter* to *Azotomonas* and *Mycobacterium* to *Mycomonas*, and upsets almost all the generic names of the red sulphur bacteria in pursuance of this plan.

Finally, Jensen nowhere gives clean-cut definition of any of his groups, and it is often difficult or impossible to draw any clear distinction between them. The outline below represents the most complete characterization we have been able to derive from a careful study of the text.

JENSEN'S CLASSIFICATION

A. Order *Cephalotrichinae*

Monotrichic or lophotrichic when flagella are present. Life energy derived mainly from oxidative processes without produc-

ing notable amounts of unoxidized split products. Water or moist earth forms, with the exception of a few peculiar plant and animal parasites. Grow poorly or not at all on artificial media. No spores formed.

1. Family Oxydobacteriaceae

Obligate aerobes, deriving their energy from direct oxidation of simple compounds of carbon, hydrogen and nitrogen. Mostly short rods, unbranched and monotrichic, except genera 4 and 7.

1. *Methanomonas.* Oxidizing methane to carbon dioxid and hydrogen
2. *Carboxydomonas.* Oxidizing carbon monoxid to carbon dioxid
3. *Hydrogenomonas.* Oxidizing hydrogen to water
4. *Acetimonas.* Oxidizing alcohol to acetic acid, form surface films
5. *Nitrosomonas.* Oxidizing ammonia
6. *Nitromonas.* Oxidizing nitrites
7. *Azotomonas.* Nitrogen fixers, branched cells occur

II. Family Actinomycetes

1. *Rhizomonas.* (*Rhizobium,* symbiotic in nodules of legumes, Lehmann and Neumann)
2. *Corynemonas.* (*Corynebacterium,* L. & N.)
3. *Mycomonas.* (*Mycobacterium,* L. & N.)
4. *Actinomyces.* (L. & N.)

III. Family Thiobacteriaceae

Colorless sulphur bacteria. Non-filamentous.

1. *Sulfomonas.* Rods, oxidize hydrogen sulfide only to sulphur
2. *Thiomonas.* Rods
3. *Thiococcus.* Cocci
4. *Thiospirillum.* Spirals

IV. Family Rhodobacteriaceae

Red sulphur bacteria. Non-filamentous.

1. *Rhodomonas.* (*Chromatium*)
2. *Rhabdomonas.* (*Rhabdochromatium*)
3. *Rhododictyon.* (*Thiodictyon*)
4. *Amoebomonas.* (*Amoebobacter*)
5. *Rhodothece.* (*Thiothece*)
6. *Rhodopolycoccus.* (*Thiopolycoccus*)
7. *Rhodococcus.* (*Thiopedia*)
8. *Lamprocystis*
9. *Rhodocystis.* (*Thiopedia*)
10. *Rhodocapsa.* (*Thiocapsa*)
11. *Rhodosarcina.* (*Thiosarcina*)
12. *Rhodospirillum.* (*Thiospirillum*)
    (Genera as defined by Winogradsky and Molisch)

V. Family Trichobacteriaceae

Filamentous water bacteria. All iron bacteria except *Cladothrix* and *Spirochaeta.*

1. *Cladothrix.* False branching, no sulphur granules
2. *Crenothrix.* No branching or sulphur granules Attached straight filaments)
3. *Beggiatoa.* Sulphur present. Straight unattached motile filaments
4. *Thiothrix.* Same as preceding but attached
5. *Leptothrix.* Unbranched straight unattached filaments. No sulphur
6. *Spirophyllum.* Flattened spiral filaments. No branching. No sulphur
7. *Spirochaeta.* Slender and flexuous spiral filaments. No branching. No sulphur

VI. Family Luminibacteriaceae

Phosphorescent and fluorescent forms. Denitrifiers.

1. *Denitromonas.* Non-liquefying, actively-dentrifying rods, usually fluorescent

2. *Liquidomonas.* Liquefiers
3. *Liquidovibrio.* Spiral forms
4. *Liquidococcus.* Liquefying cocci
5. *Solidococcus.* Non-liquefying cocci

VII. Family Reducibacteriaceae

Spiral forms, actively reducing sulphates.

1. *Solidovibrio.* Short spirals. Non-liquefying
2. *Spirillum.* Longer spirals

B. Order, *Peritrichinae*

Peritrichic when flagella are present. Rods or cocci. Not typically water bacteria. Splitting of carbohydrates or amino acids plays primary rôle in metabolism rather than oxidation or denitrification.

I. Family Acidobacteriaceae

Aerobic or facultative anaerobic. Metabolism of carbohydrates predominant, with production of acids.

1. *Denitrobacterium.* Actively-denitrifying forms
2. *Bacterium.* Rod forms which actively ferment carbohydrates and form gas. Non-liquefying
3. *Propionibacterium.* Forms which produce propionic acid. Non-motile. Do not attack casein. Form hydrogen sulphide from peptone
4. *Caseobacterium.* Attack casein. Do not form gas. Non-motile. Grow best under anaerobic conditions
5. *Streptococcus.* Chains of cocci. Usually non-liquefying. Slime formation common
6. *Micrococcus.* Irregular groups of cocci. Usually liquefying
7. Packet forms

II. Family ALKALIBACTERIACEAE

Aerobic or facultative anaerobic. Metabolism of nitrogenous compounds predominant, with production of ammonia.

1. *Liquidobacterium.* Non-spore-forming. Proteus-like bacteria
2. *Bacillus.* Usually form spores. Typically ferment carbohydrates, and reduce nitrogenous compounds with production of much ammonia. Often form diastase. Usually not indol
3. *Urobacillus.* Decompose urea and require special alkaline media for development. Usually non-liquefying

III. Family BUTYRIBACTERIACEAE

Typically anaerobic spore formers. Acting largely upon carbohydrates, forming acids, particularly butyric acid.

1. *Butyribacillus.* Acting primarily upon sugars, forming butyric acid. Usually clostridia
2. *Pectobacillus.* Acting primarily upon pectins. Usually liquefiers
3. *Cellulobacillus.* Acting primarily upon cellulose. Plectridia

IV. Family PUTRIBACTERIACEAE

Typically anaerobic spore formers. Acting largely upon proteins, bringing about putrefactive changes.

1. *Putribacillus.* Not forming an exotoxin
2. *Botulobacillus.* Producing an exotoxin. Plectridia

II. PRINCIPLES OF BOTANICAL NOMENCLATURE

The chief aim of terminology is to give an object,—in biology a kind of animal or plant—a single definite name. So important is this that both botanists and zoologists have, in international congress, established rules of nomenclature designed to attain it. Without such rules agreement as to nomenclature is impossible, and although the

rules so formulated are often arbitrary and sometimes complex, they are the result of careful study, and it is not likely that we as bacteriologists can hope to improve upon the work of trained and experienced systematists in other biological lines.

Many workers in medical bacteriology and in other special fields of applied micro-biology, who deal with only a few well-recognized species, may perhaps feel no need for any change in current practice. Few can deny, however, that it is a serious inconvenience for such names as *B. Welchii*, *B. sporogenes*, *B. perfringens*, to be used by various workers, sometimes for the same, sometimes for different organisms, or for the same form to be described as *Bacterium lactis aerogenes* or *Streptococcus lacticus* when it is isolated from milk and as *Streptococcus salivarius* or *Str. fecalis* when it is isolated from the human mouth or intestine.

When one passes from a study of the practical effects of the activity of some particular microbe to a consideration of its relationship to other forms it becomes essential, not only to have a name for each kind of organism, but to have also a system of nomenclature which will make it possible to express such relationships with reasonable clearness and accuracy.

This need is met by the Linnaean system of classification universally adopted by all biologists outside of our own limited and systematically undeveloped field. According to this system each recognizable kind of plant or animal receives a binomial Latin name, the first half designating the genus or group to which it belongs, and the second half the particular kind or species to which the name applies. The genera in turn are grouped in tribes, the tribes in families, the families in orders and the orders in classes. These divisions will often be artificial and often of course of very unequal size and importance in different groups. They make it possible, however, to express in a simple manner the essential facts of biological relationship,—the fact that A and B and C are more nearly related to each other than are any of them to D and E and F; and that the series A-F exhibits common relationships closer than any similarities which its members bear to G or H.

If such a system is accepted it is in the next place important to make sure that each group, from species to class, shall bear a single universal name. The name need not be appropriate; it need only be stable. It is an arbitrary label, not a description. If the door be once opened to criticism on the ground of inappropriateness stability of course must disappear.

It is in order to ensure uniformity and stability of nomenclature

that the International Codes referred to have been formulated; and it is to the International Rules of Botanical Nomenclature[1] (1910) that we as bacteriologists should naturally turn for guidance.

Leaving out a great many minor rules and recommendations, the most important of the rules which would affect bacteriological practice may be cited as follows.

Chap. I, Art. 7. "Scientific names are in Latin for all groups."

Chap. II, Art. 10. "Every individual plant belongs to a species (species), every species to a genus (genus), every genus to a family (familia), every family to an order (ordo), every order to a class (classis), every class to a division (divisio)."

Chap. III, Sect. 1, Art. 15. "Each natural group of plants can bear in science only one valid designation, namely the oldest, provided that it is in conformity with the rules of Nomenclature and the conditions laid down in articles 19 and 20 of Section 2."

[1] "The International Rules of Botanical Nomenclature, Adopted by the International Congresses of Vienna, 1905 and Brussels, 1910" were published in French, German, and English in the same volume by Gustav Fischer, Jena, 1912. The discussion of the 1905 Rules in Rhodora (Vol 9, no. 99, pp. 29–52, March, 1907, Preston & Rounds Co., Providence, R. I.) is more easily obtained, and serves the main needs of those interested in taxonomy.

The American Code of Botanical Nomenclature, published in the Bulletin of the Torrey Botanical Club, (Vol. 34, pp. 167–178, 1907) is used by all but one of the important botanical centers in the United States. It is a briefer, simpler set of rules formulated by an American Commission, because of certain objections to the Vienna rules. This Commission characterizes as "the height of arbitrary action" the Vienna ruling that descriptions of all new species or genera must be accompanied by a diagnosis in the Latin language. That demand was voted down also by our Society at the New Haven meeting in 1916. The contention that the "Vienna Congress failed to recognize the principle of types" affects bacterial taxonomy less than other divisions of taxonomy; type specimens cannot be preserved as with larger plants; when possible, however, the type species of a genus has been given in the following classification. The third objection of the American Commission was to the large "conservenda" list of the Vienna Congress.

The small size of bacteria, the lack of satisfactory distinguishing morphological characters, and the incompleteness and indefiniteness of the early taxonomic characterizations make it impossible to select for bacteria a workable "point of departure" (such as Species Plantarum, 1753, for flowering plants) without losing all the old classic names in the sense in which they are generally understood. Having, therefore, in this classification attempted to *conserve* these old names, it has seemed wiser to quote from the Vienna Code, especially as it is better known among bacteriologists abroad.

Chap. III, Sect. 2, Art. 19. "Botanical nomenclature begins for the different groups of plants (recent and fossil.)"

Chap. III, Sect. 2, Art. 20. "However, to avoid disadvantageous changes in the nomenclature of genera by the strict application of the rules of nomenclature, and especially of the principle of priority in starting from the dates given in Art. 19, the rules provide a list of names which must be retained in all cases. These names are by preference those which have come into general use in the fifty years following their publication, or which have been used in monographs and important floristic works up to 1890."

Chap. III, Sect. 3, Rec. III. "Orders are designated preferably by the name of one of the principal families, with the ending *ales*."

Chap. III, Sect. 3, Art. 21. "Families (familiae) are designated by the name of one of their genera or ancient generic names with the ending *aceae*."

Chap. III, Sect. 3, Art. 23. "Names of subfamilies (subfamiliae) are taken from the name of one of the genera in the group, with the ending *oideae*. The same holds for the tribes (tribus) with the ending *eae* and for the subtribes (subtribus) with the ending *inae*."

Chap. III, Sect. 3, Art. 24. "Genera receive names, substantives (or adjectives used as substantives) in the singular number and written with a capital letter which may be compared with our own family names. These names may be taken from any source whatever and may even be composed in an absolutely arbitrary manner."

Chap. III, Sect. 3, Art. 25, Rec. V. "Botanists who are publising generic names show judgment and taste by attending to the following recommendations:

"a. Not to make names very long or difficult to pronounce.

"b. Not to use again a name which has already been used and has lapsed into synonymy (homonym).

"c. Not to dedicate genera to persons who are in all respects strangers to botany or at least to natural science, nor to persons quite unknown.

[2] The date for the beginning of recognized nomenclature for the *Schizomycetes (Bacteria)* was left to be settled by the International Botanical Congress which was to have been held at London in 1915, but was postponed on account of the War. For the algae, 1753 was selected, but *six* additional later dates for various algal families &c. are added. Adoption of this classification in its final form would seem less arbitrary and much less confusing than having seven points of departure, as in the algae.

"d. Not to take names from barbarous tongues, unless those names are frequently quoted in books of travel, and have an agreeable form that is readily adapted to the Latin tongue and to the tongues of civilized countries.

"e. To recall, if possible, by the formation or ending of the name, the affinities or the analogies of the genus.

"f. To avoid adjectives used as nouns.

"g. Not to give a genus a name whose form is rather that of a subgenus or section (e. g. Eusideroxylon, a name given to a genus of Lauraceae, which, however, being valid, cannot be changed).

"h. Not to make names by the combination of two languages (nomina hybrida)."

Chap. III, Sect. 3, Art. 26. "All species, even those that singly constitute a genus, are designated by the name of the genus to which they belong, followed by a name (or epithet) termed specific, usually of the nature of an adjective (forming a combination of two names, a binomial, or binary name)."

Chap. III, Sect. 3, Art. 26, Rec. VIII. "The specific name should, in general, give some indication of the appearance, the characters, the origin, the history or the properties of the species. If taken from the name of a person, it usually recalls the name of the one who discovered or described it, or was in some way concerned with it."

Chap. III, Sect. 3, Rec. X. "Specific names begin with a small letter except those which are taken from names of persons (substantives or adjectives) or those which are taken from generic names (substantives or adjectives)."

Chap. III, Sect. 3, Rec. XIV. "In forming specific names, botanists will do well to note the following recommendations:

"a. Avoid very long names and those which are difficult to pronounce.

"b. Avoid names which express a character common to all or nearly all of the species of the genus.

"c. Avoid names taken from little known or very restricted localities, unless the species be very local.

"d. Avoid, in the same genus, names which are very much alike, especially those which differ only in their last letters.

"e. Adopt unpublished names found in traveller's notes and herbaria, attributing them to the authors concerned, only when those concerned have approved the publication.

"f. Avoid names which have been used before in the genus, or in any closely allied genus, and which have lapsed into synonymy (homonyms).

"g. Do not name a species after a person who has neither discovered, nor described, nor figured, nor in any way studied it."

"h. Avoid specific names formed of two words.

"i. Avoid names which have the same meaning as the generic name."

Chap. III, Sect. 4, No. 35. "Publication is effected by the sale or public distribution of printed matter or indelible autographs. Communication of new names at a public meeting, or the placing of names in collections or gardens open to the public, do not constitute publications."

Chap. III, Sect. 4, Art. 36. "On and after January 1, 1908, the publication of names of new groups will be valid only when they are accompanied by a Latin diagnosis."[3]

Chap. III, Sect. 4, Art. 37. "A species or a subdivision of a species, announced in a work, with a complete specific or varietal name, but without diagnosis or reference to a former description under another name, is not valid. Citation in synonymy or incidental mention of a name is not effective publication." . . . .

Chap. III, Sect. 4, Art. 38. "A genus or any other group of higher rank than a species named or announced without being characterized conformably to Article 37 cannot be regarded as effectively published (nomen nudum)." . . . .

Chap. III, Sect. 5, Art. 40. "For the indication of the name or names of a group to be accurate and complete, and in order that the date may be readily verified, it is necessary to quote the author who first published the name or combination of names in question."

Chap. III, Sect. 5, Art. 41. "An alteration of the constituent characters or of the circumscription of a group does not warrant the quotation of another author than the one who first published the name or combination of names."

Chap. III, Sect. 6, Art. 44. "A change of characters, or a revision which involves the exclusion of certain elements of a group or the addition of new elements, does not warrant a change in the name or names of a group except in cases provided for in Article 51.

Art. 45. "When a genus is divided into two or more genera, the name must be kept and given to one of the *principal* divisions,—

[3] Not accepted by this Society at the New Haven meeting, December, 1916.

either to the division containing the *type* of the original group or to the division containing the largest number of a species."

Art. 46. "When two or more groups of the same nature are united, the name of the oldest is retained."

Art. 47. "When a species or subdivision of a species is divided into two or more groups of the same nature, if one of the two forms was distinguished or described earlier than the other, the name is retained for that form."

Art. 48. "When a subgenus or section or species is moved into another genus, when a variety or other division of a species is moved into another species, retaining there the same rank, the original name of the subgenus or section, the first specific epithet, or the original name of the division of the species must be retained or must be re-established, unless, in the new position there exists one of the obstacles indicated in the articles of section 7.

Art. 49. "When a tribe becomes a family, a subgenus or a section becomes a genus, a subdivision of a species becomes a species, or the reverse of these changes takes place, and speaking generally when a group changes its rank, the earliest name (or combination of names) received by the group in its new position must be regarded as valid, if it is in conformity with the rules, unless there exist any of the obstacles indicated in the articles of section 7."

Art. 50. "No one is authorized to reject, change or modify a name (or combination of names) because it is badly chosen, or disagreeable, or another is preferable or better known, or because of the existence of an earlier homonym which is universally regarded as non-valid, or for any other motive either contestable or of little import." (See also art. 57.)

Art. 51. "Every one should refuse to admit a name in the following cases:

"1. When the name is applied in the plant kingdom to a group which has an earlier valid name.

"2. When it duplicates the name of a class, order, family or genus, or a subdivision or species of the same genus, or a subdivision of the same species.

"3. When it is based on a monstrosity.

"4. When the group which it designates embraces elements altogether incoherent, or when it becomes a permanent source of confusion or error.

"5. When it is contrary to the rules of sections 4 and 6."

Art. 53. "When a species is moved from one genus into another, its specific epithet must be changed if it is already borne by a valid species of the genus." The same rule applies to other transferred groups.

Art. 56. "In the cases foreseen in articles 51 to 56, the name to be rejected or changed is replaced by the oldest valid name in the group in question, and in default of such a one a new name must be made."

Chap. IV, Art. 58. "The rules of botanical nomenclature can only be modified by competent persons at an international congress convened for the express purpose."

In general these rules and the others not here cited seem to us entirely reasonable and well suited to bring some order into the chaos of bacteriological nomenclature, as they have done in other botanical fields. The requirement of a Latin diagnosis seems, however, an *unnecessary* one, and it is certainly a requirement which would not be readily accepted by working bacteriologists.

The question as to the date at which valid bacteriological nomenclature shall be considered to begin is one of fundamental importance in the application of this system. As noted above, the International Botanical Congresses have established such dates for the various groups of higher plants, but not as yet for the bacteria. Erwin F. Smith (1905), in his suggestive discussion of the general problems of bacterial classification, urges that all names suggested prior either to 1872 (the date of publication of Cohn's classic paper) or to 1881 (the date of the introduction of solid culture media) should be considered *nomina nuda.* With the latter suggestion we are in general in accord. The characterizations of bacteria before this date were almost of necessity based upon mixed cultures, and the descriptions are vague and puzzling to a degree. Furthermore, the acceptance of these earlier descriptions would rob us of almost all our familiar names, and give us strange generic and specific names which it would be impossible ever to force upon workers in applied bacteriology. The introduction of a simple method of making pure cultures marks a natural point of departure for bacterial classification; but a slightly later date, 1885, seems to form a better point of departure, since the important contribution which appeared at that time, Zopf's Spaltpilze, established a group of bacterial genera which are for the most part natural ones, and which bear names familiar to all bacteriologists down to the present day.

We therefore recommend to the Society of American Bacteriologists:

(a) That the International Rules for Botanical Nomenclature be accepted by the Society as governing bacterial terminology, with the exception that French, English or German may be substituted for Latin in the diagnosis.

(b) That the date of publication of the third edition of Zopf's Spaltpilze be considered as the date for the beginning of bacteriological nomenclature for the purpose of determining priority, with the exception of a list of genera conservanda to be adopted by the Society at its 1918 meeting.

(c) That the Society take steps to present these recommendations to the next International Botanical Congress, and if possible to secure favorable action thereon by that body.

III. SYSTEM OF CLASSIFICATION RECOMMENDED BY THE COMMITTEE: FAMILIES

It has seemed evident to the Committee that none of the systems of classification outlined above gives a satisfactory picture of the real relationships of bacterial types. The classifications by Zopf and by Lehmann and Neumann are good as far as they go; these earlier classifications might be helpfully supplemented by Kruse's groups of bacilli and by the Winslows' genera of cocci. Many of the distinctions in Jensen's outline are suggestive and important. Each of the general systems proposed has, however, been limited in important respects. The earlier workers ignored physiological differences, while Jensen gave to certain of them a quite arbitrary and artificial importance. We are of the opinion that the facts at hand warrant a review of the whole question, and the working out of a new system of classification which shall include what is valid and discard what is arbitrary in the older classifications—with no idealistic conceptions, either morphological or physiological in mind—but with the sole purpose of recognizing and defining the principal groups of bacteria which exhibit circumstantial evidence of common evolutionary relationship.

The conditions which call for reform, and the principles which should govern them, have been admirably stated by Jordan (1914) as follows:

The present nomenclature of bacteriology may be criticized on two grounds: first, as already pointed out, for the unwieldy size that certain "genera" have been allowed to assume; and, second, for the haphazard way in which trinomial and even quadrinomial names have been bestowed. Such names can be properly employed only with reference to subspecies or varieties; and designations like *B. coli communis*, *Granulobacillus saccharobutryricus mobilis non-liquefaciens*, and *Micrococcus acidi paralactici liquefaciens Halensi*, are both cumbersome and unscientific. The use of a single genus name for a multitude of organisms is, in fact, responsible for the tendency toward trinomial nomenclature, and the remedy for both conditions would seem to lie in the abandonment of such a term as *Bacillus* for the name of a genus and the frank establishment of new genera on the basis of physiologic characters: such, for example, as distinguish the colon-typhoid group or the diphtheria group of bacilli. Until some such reform in nomenclature is brought about the names used to designate different kinds of bacteria will fail to make clear the group relationships which undoubtedly exist, and will continue to be a stumbling block to all students of the subject.

The first point which must be considered in formulating a system of bacterial classification is the limitation of the general field to be considered—the definition of the class Schizomycetes. There are three groups in particular whose relationships are doubtful, the Myxobacteriaceae, the *Sporothrix* forms, and the Spirochaetes.

The Myxobacteriaceae are included by Smith (1905) as a family of the Schizomycetes, but we cannot feel that such a step is justified. The peculiar life cycle of these forms with their amoeboid stage and rather complex cyst production seems to separate them quite widely from the other bacteria. They should certainly constitute a distinct order, the Myxobacteriales but they may conveniently be included within the class Schizomycetes.

The *Sporothrix* forms are much more sharply differentiated from the bacteria by their much-branched septate mycelium and their characteristic spores. They undoubtedly belong with the higher fungi.

The position of the Spirochaetes is much more uncertain. The character of the disease processes excited by these organisms as well as their relation to certain chemicals and the presence in certain forms of the so-called "undulating membrane," suggest a protozoan nature. The character of the flagella, the rapid multiplication by transverse division and the behavior of these organisms in plasmolysis are on the other hand distinctively bacterial characters. It is certainly a straining of the facts to place these forms in the family Spirillaceae, as if they were closely related to *Vibrio* and *Spirillum*. The best course seems to be to consider them as forming a distinct family intermediate between the bacteria and the Protozoa.

The next problem to be considered concerns the relation between the higher filamentous bacteria and the ordinary types. We are inclined in this matter to follow Zopf and the earlier workers in separating these organisms rather widely, rather than Kruse and Jensen who group them together. The red and the colorless sulphur bacteria and the iron bacteria differ fundamentally in metabolism from the ordinary bacteria; and these types as well as the *Cladothrix* forms have a quite characteristic morphology and habitat.

Almost all systematists have separated the purple bacteria from the ordinary types, and both Zopf and Migula gave the other filamentous forms at least distinct family rank. The classification most commonly adopted is that of Migula, which separates the sulphur bacteria as a distinct order and includes the iron bacteria, the *Cladothrix* types, and even the branched Sphaerotilus forms with the ordinary bacteria, but in a separate family, Chlamydobacteriaceae. It seems to us that the broad difference between all these filamentous oxidative water bacteria and the types we cultivate in the laboratory is far more fundamental than that between, for example, the spherical and the rod-shaped bacteria. We suggest that the filamentous types, which are not sulphur users, should be placed in the Chlamydobacteriales, defined as below, while the sulphur forms should be placed in a separate order, Thiobacteriales. In this we follow Buchanan (1917).

We have not attempted to discuss the genera of these two series, as they are beyond the scope of the work of the ordinary bacteriologist. The orders Chlamydobacteriales and Thiobacteriales as defined belong largely to the botanist, the group of the Eubacteriales with its cultivable forms, studied by a special physiological technique, to the bacteriologist.

Jensen's division into the orders Cephalotrichinae and Peritrichinae places the filamentous bacteria with the oxidizing bacteria, the Actinomycetes and the phosphorescent and reducing forms, and separates all these groups from the bacteria which possess peritrichic flagella and utilize carbohydrates and amino-acids. There are undoubtedly real differences between Jensen's two main groups, but we are inclined to the opinion that the first, second, sixth and seventh families of his Cephalotrichinae are much more closely related to the Peritrichinae than to the filamentous water bacteria, and that the differences in metabolism and arrangement of flagella on which he bases his division are of family rather than ordinal rank.

Taking the order Eubacteriales as including all the Schizomycetes except the *Cladothrix*, *Crenothrix*, *Beggiatoa* and purple bacteria types, the next problem is a division of this order into natural family groups.

Of the three classic bacterial families, the Coccaceae, the Spirillaceae, and the Bacteriaceae, the first two seem to us to represent fairly distinct entities which should be preserved intact. They appear in the classifications of Hueppe, Lehmann and Neumann, Migula, and Kruse, and the breaking up of the distinction between these types and the rod-shaped bacteria by Jensen seems to us a distinct step backward. It is not of course the single morphological character of roundness or twistedness that is so important, but the fact that both the cocci and the spiral bacteria (except the Spirochaetes) show a number of common characters which appear to us to indicate real relationship.

The Actinomycetes group, established by Lehmann and Neumann for the diphtheria, tuberculosis and actinomyces types, seems to us a good family,—characterized by the branching of the cells, frequent occurrence of clubbed forms, the ab-

sence of motility and the rather slow, often mold-like growth on media. This family was properly named Mycobacteriaceae by Chester. The fusiform bacilli may be placed here, for the present, at least.

The classic family Bacteriaceae seems to us to be the group which needs most radical revision. It includes a great complex of organisms differing from each other radically in structure and metabolism, and might conveniently be split up into at least five distinct families.

First of all it seems to us that the group of the spore formers, distinguished as the genus *Bacillus* by Zopf, is a sufficiently clearly marked group to deserve family rank. The power of spore formation in itself is a rather fundamental one, and its combination with a very complex metabolism, Gram-positive staining, and the peritrichic arrangement of flagella, when present, marks off the Bacillaceae as one of the highest groups of the bacteria.

The next group of rod-shaped organisms which deserves special recognition is the group characterized by a very primitive metabolism,—life energy being derived mainly from simple oxidative processes without the splitting of carbohydrates or the metabolism of amino-acids. This is Jensen's Oxydobacteriaceae, including the types which oxidize methane, carbon monoxid and hydrogen, the acetic acid bacteria, the nitrosifiers and nitrifiers, and the *Azotobacter* type. Unfortunately the name given by Jensen cannot stand in face of the rule of botanical nomenclature that a family name must be formed from one of its component genera with the suffix *aceae*. We suggest the name Nitrobacteriaceae for this family.

A third group of rod-shaped bacteria which seems to us worthy of family rank is the group which roughly corresponds to the rod forms in Jensen's family, Luminibacteriaceae, with the addition of other types, not fluorescent or phosphorescent, which seem closely allied to the former. This group should provisionally include all the forms (except certain genera of Nitrobacteriaceae) which possess polar flagella, this primary characteristic being associated as a rule with a limited carbohydrate metabolism

and active oxidative action. The common types of this family are either water bacteria or plant pathogens. The family should be called *Pseudomonadaceae* from Migula's genus.

A fourth family should perhaps be recognized for the high acid formers of the *B. acidophilus* and *B. bifidus* and *B. bulgaricus* types. The peculiar morphology and the unique acid tolerence of these organisms seem to warrant family rank, and we suggest the generic name *Lactobacillus* and the family name Lactobacillaceae.

The rest of the rod-shaped bacteria may for the present most wisely be left in the family Bacteriaceae. The typical forms in this family are those of the compact colon-typhoid group, with their peritrichic flagella and complex metabolic powers. The *B. cloacae* types, the *Proteus* forms and the peritrichic water bacteria grade through *B. aerogenes* into the colon-typhoid series. Jensen's groups of the *Acido-* and *Alkalibacteriaceae* do not seem to us of family rank.

The family Bacteriaceae must necessarily remain somewhat of an *omnium gatherum* from which other families will no doubt be split off as knowledge advances. We have hesitated however to burden the literature with unnecessary terminology, and prefer to leave these forms in the family Bacteriaceae until further study indicates that a sharper differentiation is desirable.

IV. SYSTEM OF CLASSIFICATION RECOMMENDED BY THE COMMITTEE: GENERA

We may now proceed to a brief review of the eight families proposed, and of their generic subdivisions.

The Nitrobacteriaceae are clearly the most primitive of the Eubacteriales. Their power to live without complex organic substances would have made it possible, as Jensen points out, for them to flourish at a very early period in the world's history, and their simple structure is in harmony with the view that they represent the ancestral type of all other bacteria.

The three genera suggested by Jensen for the types which derive their life energy from the oxidation of methane, carbon monoxid and hydrogen respectively, include only a few rare and

little-studied forms. Their metabolism is, however, so unique as perhaps to warrant the recognition of the genera, *Methanomonas*, *Carboxydomonas* and *Hydrogenomonas*. His four other genera, which respectively include the acetic acid bacteria, the nitrosifiers, the nitrifiers and the nitrogen fixers of the soil, are clearly distinct genera; but they should bear the old names, *Mycoderma*, *Nitrosomonas*, *Nitrobacter*, and *Azotobacter*, not the new names unwarrantably coined by Jensen.

The second family to be considered is that of the Mycobacteriaceae, including a series of organisms which show a definite tendency to branch, leading finally to the complex mycelia of *Actinomyces*, which exhibit marked peculiarities in staining reactions, and which show in the three most typical genera a peculiar kind of localized pathogenicity leading to the production of local swellings of host tissue.

All of these organisms are strongly aerobic and oxidative forms, and *Actinomyces* can derive its nitrogen from nitrates, although not from free nitrogen. These are primitive characteristics, but the Gram-positive staining and the difficult growth of the pathogenic types on media indicate a considerable degree of differentiation. Theobald Smith has pointed out that the tubercle bacillus represents a peculiarly high type of parasitic adaptation, which is natural if it is derived, as we believe, from one of the earliest bacterial groups.

We may perhaps consider *Rhizobium* as connecting the Nitrobacteriaceae and Mycobacteriaceae, *Rhizobium* deriving its origin from *Azotobacter*. In Mycobacteriaceae we may place Lehmann and Neumann's genus, *Mycobacterium*, including the tubercle and pseudotubercle forms, the leprosy organism, and possibly the glanders bacillus. This organism is commonly classed with the tubercle bacillus, which it resembles in its branching cells and slow growth on media and in certain phases of its pathogenesis. Its Gram-negative staining reaction is a peculiar feature in this otherwise Gram-positive group. This genus clearly is very close to *Actinomyces*, which in turn forms a connecting link with the higher molds, as indicated by the tendency to branching and the formation of conidia.

The genus, *Corynebacterium*, including the diphtheria and diphtheroid groups, is related to this family by the tendency to cell branching and irregular staining, by its positive Gram reaction, and its oxidative tendencies. On another side it is allied in many respects with the streptococci, and very possibly may have formed a connecting link with this group.

With some doubts, we have placed the fusiform bacilli in this group as the genus *Fusiformis* (Holling). They resemble the members of the family Mycobacteriaceae in their tendency to filament formation and irregular staining, in their Gram-positive reaction and lack of motility. They of course differ markedly in their anaerobic and essentially putrefactive metabolism; but seem on the whole to fit better here than anywhere else.

Another distinctly primitive family is that of the Pseudomonadaceae. The polar flagella, the oxidative metabolism and active production of ammonia with very limited utilization of carbohydrates, and the predominant water habitat are all primitive characteristics. Both the fluorescent water bacilli and many plant pathogens like *Ps. campestris* may for the present be grouped in a single genus. If no limit were recognized for the beginning of adequate bacteriological nomenclature the name of this genus, as pointed out by Smith (1905), must be *Bacterium*, for both Ehrenberg (1838) and Cohn (1872) used this term for a form with terminal flagella. If, however, names proposed prior to 1881 are considered as *nomina nuda* it is proper to use the term Bacterium as defined by Zopf for the main group of non-spore-forming bacteria, and Migula's later differentiation of the types with polar flagella becomes valid. This genus, it may be noted, includes one form which in a limited degree is pathogenic for man, *Ps. pyocyanea*.

The next family to be considered is that of the spiral bacteria (excluding the Spirochaetes). The vibrios and spirilla, by their polar flagella, simple metabolism, (primarily oxidative, with formation of ammonia, rather than fermentative) and water habitat, are clearly allied to the *Pseudomonas* types.

They form two fairly distinct morphological genera, *Vibrio*, which includes the comma forms with a single terminal flagellum, and *Spirillum*, which includes the large lophotrichic water spirilla. Migula (1896) calls the first of these genera *Microspira*, Schroeter. Smith (1905) has pointed out that if no time limit be placed on bacterial terminology, Trevisan's name *Pacinia* antedates *Microspira* and must supplant it. If, on the other hand, we exclude names suggested prior to 1881 Zopf's use of the name *Vibrio* will stand.

We see no good reason for recognizing a non-motile genus, *Spirosoma*, as Migula does, the loss of motility being presumably a character of very slight importance here as in other bacterial groups. It is evident that the spirochaetes are quite widely separated by structure and parasitic habit from the other genera of the spiral bacteria. It may well be that these forms do not belong among the bacteria at all. We have placed them for the present in a separate family at the end of our system.

We come next to the spherical bacteria, which make up a fairly well-defined family including two main series, one saprophytic, the other parasitic. The first is allied, through the water micrococci, to *Pseudomonas*, and the second, through the streptococci, to *Corynebacterium* and possibly to the colon-typhoid group.

In the subdivisions of this family we have, for the most part, followed the revision of the Winslows (1908) with the changes in terminology suggested by Buchanan (1915). We are inclined to think, however, that in the light of more recent investigations the genus *Diplococcus* as defined by the Winslows is an artificial one. It seems to us that the pneumococcus is more closely allied to the streptococci than it is to the Gram-negative, biscuit-shaped gonococci and meningococci. We are also doubtful of the validity of the genus, *Ascococcus* (*Leuconostoc*). For the present we have omitted it from our scheme, and we have re-defined *Streptococcus* so as to include the pneumococci and the zooglea-forming types, as well as the true chain formers.

Following the Winslows, the white staphylococci, characterized by more abundant surface growth and less vigorous gelatin liquefaction, as well as by the lack of orange pigment, are separated from *Staphylococcus* in a separate genus, *Albococcus*. Among the Gram-negative, feebly fermenting saprophytic forms, the yellow types are placed in *Micrococcus* and *Sarcina*, the red types in *Rhodococcus*. The mere presence or absence of packets, as the Winslows point out, is a slender basis for a distinction between the first two of these genera, but it is perhaps best to retain a name which has nearly a century of usage behind it until more definite proof of its invalidity is forthcoming.

The seventh family, the Bacteriaceae, must for the present include all the non-spore-forming, Gram-negative rods which when motile possess peritrichic flagella. Besides several lesser genera, there are two principal series here, one parasitic and often pathogenic, the other saprophytic. The first group, which may conveniently bear the old generic name *Bacterium*, is the colon-typhoid-dysentery group.

If the names of organisms imperfectly described in the early days of bacteriology were to be accepted as valid the name *Bacterium*, as pointed out above, would have to be reserved for the forms with polar flagella, leaving the great mass of non-spore-forming bacteria to be designated by some new and unfamiliar name, or by some very old and equally unfamiliar one which might be dug out of the literature. The fact that this group includes many of the common pathogenic forms universally designated by medical workers under one of the names *Bacillus* or *Bacterium*, would make it peculiarly unfortunate to introduce new terminology. If, however, we consider names proposed prior to 1885 as *nomina nuda*, the use by Zopf of the term *Bacterium* for the non-spore-formers becomes valid and it is permissible to retain this name for the group under consideration. *B. abortus* may for the present be left in this genus in spite of its peculiar oxygen relations. Here also the proteus and aerogenes series and the chromogenic water bacteria may be left for the present.

On the other hand the plant pathogens, such as *B. amylovorus*, *B. phytophthorus*, *B. caratovorus*, *B. melonis*, and *B. solanacearum* seem to us perhaps to deserve distinct generic rank. They resemble the proteus group in their Gram-negative reaction, general growth characters, tendency to slime formation, and generally negative indol reaction, but their fermentative powers are less vigorous and the exact biochemical changes they produce in carbohydrates are unknown. We suggest that all the peritrichiate plant pathogens be for the present grouped together under a new genus to be called *Erwinia*, after our American Pasteur of bacterial plant pathology, Erwin Smith.

Two other groups of the Bacteriaceae seem to us to deserve distinct generic rank. The hemorrhagic septicemia organisms by their bipolar staining and characteristic pathogenesis may properly be grouped under the name *Pasteurella*, including in the genus the causative organisms of fowl cholera, swine plague, rabbit septicemia, hemorrhagic septicemia of cattle and bubonic plague.

The other group, for which we suggest the generic name, *Hemophilus*, should include the small non-motile parasites, which will grow only on media in the presence of blood or other body fluids. The influenza bacillus, the Koch-Weeks bacillus and the Bordet-Gengou bacillus are the principal representatives of this group.

The peculiarities of the high acid-forming Gram-positive bacteria of the *B. bulgaricus*, *B. acidophilus* and *B. bifidus* types seem so significant as to warant the creation for them of a distinct family, the *Lactobacillaceae*.

Finally, the last and probably the most complex family is that of the Bacillaceae, which includes the large Gram-positive rods which form endospores and actively decompose protein media through the agency of enzymes. These organisms are clearly related to each other, but include two distinct series, one aerobic, the other anaerobic. The aerobic types of the *B. subtilis* and *B. megatherium* series, with *B. anthracis* and B. *lactimorbi*, may properly bear the ancient name *Bacillus* applied to them by Zopf, while the anaerobic, rod-shaped types

should properly bear the name *Clostridium* applied by the same author to those of them which showed a spindle-shaped enlargement at spore formation. It should be understood, however, that we define the genus by its anaerobic properties, and include in it plectridium and clostridium forms and those which show no enlargement at spore formation. It is quite possible that this group may later be subdivided, but we prefer to err on this side rather than unduly to complicate terminology. The bacillus of malignant oedema may for the present be left in this genus, although it is said to show a Gram-negative reaction.

In regard to the genera to be recognized in the family Spirochaetaceae—which, as pointed out above, must be considered an aberrant group intermediate between bacteria and protozoa—we have consulted with Prof. G. N. Calkins of Columbia, and in the outline below have accepted the characterization of the four genera, *Spirochaeta*, *Cristispira*, *Saprospira*, and *Treponema* as given by him.

The families and genera listed below seem to us to represent the minimum number of bacterial groups which must be recognized in order to embody our present knowledge of bacterial relationships; and we submit it in the hope that it may serve as a basis for the future development of classifications which shall more and more closely approximate the facts of evolutionary history.

It is our hope that the present report of the Committee may be put in type and distributed to the members of the Society in proof form for careful consideration. After all suggestions in connection with it have been fully considered a revised report may be presented next year for formal adoption.

If this plan can be carried out we earnestly bespeak the support of the individual members for such a modification of the classification here presented as the Society may finally adopt. It will of course be subject to constant change with advancing knowledge; but such changes should be based on careful systematic study and carried out in accordance with recognized rules of nomenclature. A single clearly defined system of families and genera must form the starting point for progress.

Such a system the Society should be able to agree upon after a year's consideration. A formal endorsement will be of little value, however, unless the names suggested are used in actual practice, in written papers and in the teaching of the class-room. It is easy to continue in the old slipshod ways, and there will be a powerful force of inertia to overcome. If, however, this Society can actually bring into use a system of classification which approximately represents natural bacterial relationships and terminology which is uniform and in accord with the rules of priority, a great service will have been rendered to the science of bacteriology.

V. SUGGESTED OUTLINE OF BACTERIAL CLASSIFICATION

THE CLASS SCHIZOMYCETES

Minute, one-celled, chlorophyll-free, colorless, rarely violet-red or green-colored plants, which typically multiply by dividing in one, two or three directions of space, the cells thus formed sometimes remaining united into filamentous, flat, or cubical aggregates. Filamentous species often surrounded by a common sheath. Capsule or sheath composed in the main of protein matter. The cell plasma generally homogeneous without a nucleus. Sexual reproduction absent. In many species resting bodies are produced, either endospores or gonidia. Cells may be motile by means of flagella.

A. ORDER MYXOBACTERIALES[4]

Cells united during the vegetative stage into a pseudoplasmodium which passes over into a highly-developed cyst-producing resting stage.

B. ORDER THIOBACTERIALES[4]

Cells free or united in elongated filaments. Water forms, not easily cultivable. Life energy derived mainly from oxidative

[4] These first three orders are included briefly to give the complete setting of the fourth, the Eubacteriales, with which we are primarily concerned.

processes. Cells typically containing either granules of free sulphur or bacterio-purpurin or both, usually growing best in the presence of hydrogen sulphide.

C. ORDER CHLAMYDOBACTERIALES[4]

Cells normally united in elongated filaments. Sulphur and bacterio-purpurin are absent. Iron often present and usually a well-marked sheath.

D. ORDER EUBACTERIALES. Ordo nov.

Synonyms: *Bacterina* Perty 1852 in part; *Eubacteria* Schroeter 1886; *Eubacteriaceae* A. J. Smith 1902.

The order Eubacteriales includes the forms usually termed the true bacteria, that is, those forms which are considered least differentiated and least specialized. The cell metabolism is not primarily bound up with hydrogen sulphide or other sulfur compounds, the cells in consequence containing neither sulfur granules nor bacterio-purpurin. The cells apparently do not possess a well-organized or well-differentiated nucleus. The cells are usually minute and spherical, rod-shaped or spiral in shape, in most genera not producing true filaments; the filaments when formed not sheathed, and frequently branching, thus being differentiated from the iron bacteria. The cells may occur singly, in chains or other groupings. The cells may be motile by means of flagella, or non-motile; they are never *notably* flexuous. Cell multiplication occurs always by transverse, never by longitudinal fission. Some genera produce endospores, particularly the rod-shaped types. More or less branching of cells and filaments may occur, reaching its maximum expression in the genera *Nocardia* and *Actinomyces* which may show typical mycelium formation, intergrading with the molds. Chlorophyll is absent, though the cells may be pigmented. The cells may be united into gelatinous masses, but never form motile pseudoplasmodia nor develop a highly specialized cyst-producing fruiting stage, such as is characteristic of the *Myxobacteriales*.

FAMILY I. NITROBACTERIACEAE Fam. nov.

Organisms usually rod-shaped (sometimes spherical in *Nitrosomonas* and possibly in *Azotobacter*). Cells motile or non-motile; when motile with polar, never peritrichous, flagella. Endospores never formed. Obligate aerobes, capable of securing growth energy by the direct oxidation of carbon, hydrogen or nitrogen or of simple compounds of these. Non-parasitic (usually water or earth forms).

1. *Hydrogenomonas* Jensen 1909

Monotrichic short rods capable of growing in the absence of organic matter, and securing growth energy by the oxidation of hydrogen (forming water). Kaserer (1905) who first described the organism states that his species will also grow well on a variety of organic substances.

The type species is *Hydrogenomonas pantotropha* (Kaserer) Jensen. Nikleuski (1910) described two additional species, *H. vitrea* and *H. flava*.

2. *Methanomonas* Jensen 1909

Monotrichic short rods capable of growing in the absence of organic matter and securing growth energy by the oxidation of methane (forming carbon dioxide an dhydrogen). The type species is *Methanomonas methanica* (Söhngen) Jensen.

3. *Carboxydomonas* Jensen 1909

Autotrophic rod-shaped cells capable of securing growth energy by the oxidation of carbon monoxide (forming carbon dioxide). The type species, *Carboxydomonas oligocarbophila* (Beijerinck and van Delden) Jensen, is described as non-motile.

4. *Mycoderma* Persoon 1822 emended

Synonyms: Ulvina Kuetzing 1837; *Umbina* Naegeli 1849; *Bacteriopsis?* Trevisan 1885; *Gliacoccus?* Maggi 1886; *Acetobacter* Fuhrmann 1905; *Acetimonas* Jensen 1909.

Cells rod-shaped, frequently in chains, non-motile. Cells grow usually on the surface of alcoholic solutions, securing growth energy by the oxidation of alcohol to acetic acid. Also capable of utilizing certain other carbonaceous compounds, as sugar and acetic acid. Elongated, filamentous, club-shaped, swollen and even branched cells common and quite characteristic.

The type species is *Mycoderma aceti* Thompson?

5. *Nitrosomonas* Winogradsky 1892

Includes *Nitrosococcus* Winogradsky 1892.

Cells rod-shaped or spherical, motile or non-motile, if motile with polar flagella. Capable of securing growth energy by the oxidation of ammonia to nitrites. Growth on media containing organic substances scanty or absent.

The type species is *Nitrosomonas europoea* Winogradsky.

6. *Nitrobacter* Winogradsky? 1892

Synonym: Nitrosobacterium? Rullmann 1897.

Cells rod-shaped, non-motile, not growing readily on organic media or in the presence of ammonia. Cells capable of securing growth energy by the oxidation of nitrites to nitrates.

Winogradsky named no species, although he described one. It might be termed ***Nitrobacter Winogradskyi*** and made the type species.

7. *Azotobacter* Beijerinck 1901

Synonyms: Parachromatium Beijerinck 1903; *Azotomonas* Jensen 1909.

Relatively large rods, or even cocci, sometimes almost yeast-like in appearance, dependent primarily for growth energy upon the oxidation of carbohydrates. Motile or non-motile; when motile, with tuft of polar flagella. Obligate aerobes usually growing in a film upon the surface of the culture medium. Capable of fixing atmospheric nitrogen when grown in solutions

containing carbohydrates and deficient in combined nitrogen. The best-known free-living nitrogen-fixing bacteria of the soil.

The type species is *Azotobacter chroöcoccum* Beijerinck.

8. *Rhizobium* Frank 1889

Synonyms: Phytomyxa Schroeter 1886; *Cladochytrium* Vuillemin 1888; *Rhizobacterium* Kirchner 1895; *Pseudorhizobium* Hartleb 1900; *Rhizomonas* Jensen 1909.

Comment. Phytomyxa Schroeter has priority over *Rhizobium*, but because of the confusion which would arise from the substitution of the older correct name for the better known term *Rhizobium*, the committee recommends the adoption of the latter.

Minute rods, motile when young by means of polar flagella. Involution forms abundant and characteristic when grown under suitable conditions. Obligate aerobes, capable of fixing atmospheric nitrogen when grown in the presence of carbohydrates in the absence of compounds of nitrogen. Produce nodules upon the roots of leguminous plants.

The type species is *Rhizobium leguminosarum* Frank.

FAMILY II. MYCOBACTERIACEAE Chester 1897

Cells usually elongated, frequently filamentous and with a decided tendency to the development of branches, in some genera giving rise to the formation of a definite branched mycelium. Cells frequently show swellings, clubbed or irregular shapes. Endospores not produced, but conidia developed in some genera. Usually Gram-positive. Non-motile. Many species are parasitic in animals or plants. Complex proteins usually required. As a rule strongly aerobic, (except for some species of *Actinomyces* and the genera *Fusiformis* and *Leptotrichia*), and oxidative. Growth on culture media often slow; some genera show mold-like colonies.

1. *Actinomyces* Harz 1877

Synonyms: Streptothrix Cohn 1875, not *Streptothrix* Corda 1839; *Discomyces* Rivolta and Micellone 1878; *Micromyces* Gruber 1891, not

Micromyces Dangeard 1888; *Oöspora* Sauvageau and Radais 1892; not *Oöspora* Wallroth 1833; *Cohnistreptothrix* Pinoy 1913.

Organism growing in form of a much-branched mycelium, which may break up into segments that function as conidia. Usually parasitic, with clubbed ends of radiating threads conspicuous in lesions in animal body. No *aerial* hyphae or conidia. Some species are microaerophilic or anaerobic. Non-motile.

The type species is *Actinomyces bovis* Harz.

2. *Nocardia* Trevisan 1889

Synonyms: Actinomyces of many authors; *Streptothrix* of many authors; *Thermoactinomyces* Tsilinsky 1899.

Branched filaments, resembling a mycelium, readily breaking up into segments, usually saprophytic soil forms. Differs primarily from *Actinomyces* in the development of *aerial* hyphae and conidia. Usually aerobic. Many are pigment formers. Colonies as a rule mold-like on culture media.

3. *Mycobacterium* Lehmann and Neumann 1896

Synonyms: Sclerothrix Metschnikoff 1888, not *Sclerothrix* Kuetzing 1849; *Coccothrix* Lutz 1886; *Mycomonas* Jensen 1909.

Slender rods which are stained with difficulty, but when once stained are acid-fast. Cells sometimes show swollen, clavate or cuneate forms, and occasionally even branched filaments. Non-motile, Gram-positive. No endospores. Growth on media slow. Aerobic. Several species pathogenic to animals.

The type species is *Mycobacterium tuberculosis* (Koch) Lehmann and Neumann.

4. *Corynebacterium* Lehmann and Neumann 1896

Synonyms: Corynemonas Jensen 1909; *Corynethrix* Bingert 1901.

Slender, often slightly curved, rods with tendency to club formation, branching cells occasionally seen in old cultures. Barred irregular staining. Not acid-fast. Gram-positive. Non-

motile. Aerobic. No endospores. Some pathogenic species produce a powerful exotoxin. Characteristic snapping motion is exhibited when cells divide.

The type species is *Corynebacterium diphtheriae* (Loeffler) Lehmann and Neumann.

5. *Fusiformis* Hoelling 1910

Synonym: Mantegazzaea Vuillemin 1913, not *Mantegazzaea* Trevisan 1879.

Obligate parasites. Cells usually elongate and fusiform, staining somewhat irregularly. Filaments sometimes formed; non-branching. Non-motile. No spores. Growth in laboratory media feeble.

The type species (?) is *Fusiformis termitidis* Hoelling.

6. *Leptotrichia* Trevisan 1879 emended

Synonyms: Leptothrix Robin 1847, not *Leptothrix* Kuetzing 1843; *Rasmussenia* Trevisan 1889.

Thick, long, straight or curved threads, frequently clubbed at one end and tapering to the other. Gram-positive when young. Threads fragment into short, thick rods. Anaerobic or facultative. Non-motile. Filaments sometimes granular; non-branching. No aerial hyphae or conidia. Parasites or facultative parasites.

The type species is *Leptotrichia buccalis* (Robin) Trevisan.

FAMILY III. PSEUDOMONADACEAE

Short rods, usually motile. Flagella single, polar. Gram-negative. Not obligate aerobes. Many species active ammonifiers. Many species produce water-soluble pigments or green fluorescence; yellow pigment common. Some species are photogenic. Soil and water bacteria, with many plant parasites.

1. *Pseudomonas* Migula 1894

Synonyms: Bactrillum Fischer 1895; *Arthrobactrimium* Fischer 1895; *Arthrobactrillum* Fischer 1895; *Eupseudomonas* Migula 1895; *Bactrinius* Kendall 1902; *Bactrillius* Kendall 1902; *Bacterium* Ehrenberg emended E. F. Smith 1905; *Denitromonas* Jensen 1909; *Liquidomonas* Jensen 1909.

Rod-shaped, short, usually motile by means of polar flagella or rarely non-motile. Aerobic and facultative. Frequently gelatin liquefiers and active ammonifiers. No endospores. Gram stain variable, though usually negative. Fermentation of carbohydrates as a rule not active. Frequently producing a water-soluble pigment which diffuses through the medium as green, blue, purple, brown, etc. In some cases a non-diffusible yellow pigment is formed. Many yellow species are plant parasites.

FAMILY IV. SPIRILLACEAE Migula 1894

Cells elongate, more or less spirally curved. Cell division always transverse, never longitudinal. Cells non-flexuous. Usually without endospores. As a rule motile by means of polar flagella, sometimes non-motile. Typically water forms, though some species are intestinal parasites.

1. *Vibrio* Miller 1773 emended E. F. Smith 1905

Synonyms: Pacinia Trevisan 1885; *Microspira* Schroeter 1886; *Pseudospira* DeToni and Trevisan 1889; *Liquidovibrio* Jensen 1909; *Solidovibrio* Jensen 1909; *Photobacterium?* Beijerinck 1889.

Cells short bent rods, rigid, single or united into spirals. Motile by means of a single (rarely two or three) polar flagellum, which is usually relatively short. Many species liquefy gelatin and are active ammonifiers. Aerobic and facultative. No endospores. Usually Gram-negative. Water forms, a few parasites.

The type species is *Vibrio cholerae* (Koch) Buchner.

2. *Spirillum* Ehrenberg 1830 emended Migula 1894

Synonyms: Spirobacillus? Metschnikoff 1889; *Spirosoma* Migula 1894; *Sporospirillum?* Jensen 1909.

Cells rigid rods of various thicknesses, length, and pitch of the spiral, forming either long screws or portions of a turn. Cells motile by means of a tuft of polar flagella (5 to 20) which are mostly half circular, rarely wavy-bent. These flagella occur on one or both poles; their number varies greatly and is difficult to determine, since in stained preparations several are often united into a common strand. Endospore formation has been reported in some species. Habitat; water or putrid infusions.

FAMILY V. COCCACEAE Zopf 1884 emended Migula 1894

Synonyms: Sphaerobacteria Cohn 1872; *Coccogenae* Trevisan 1885; *Coccacei* Schroeter 1886; *Coccobacteria* Schroeter 1886.

Cells in their free conditions, spherical; during division somewhat elliptical. Division in one, two or three planes. If the cells remain in contact after division they are frequently flattened in the plane of division. Motility rare. Endospores absent. Metabolism complex, usually involving the utilization of amino-acids or carbohydrates.

Tribe 1. Streptococceae Trevisan

Parasites (thriving only or best on or in the animal body) Grow well under anaerobic conditions. Many forms grow with difficulty on media, none very abundantly. Planes of fission usually parallel, producing pairs or short or long chains, never packets. Generally stain by Gram. Produce acid but no gas in glucose and lactose broth. Pigment, if any, white or orange.

1. *Neisseria* Trevisan 1885

Synonyms: Diplococcus Weichselbaum 1886 in part; *Gonoccoccus?* Neisser? 1879; *Merismopedia* Zopf 1885, not *Merismopedia* Meyen 1839.

Strict parasites, failing to grow or growing very poorly on artificial media. Cells normally in pairs of flattened cells. Gram-negative. Fermentative powers low. Growth fairly abundant on serum media, usually whitish or yellowish.

The type species is *Neisseria gonorrhoeae* Trevisan.

2. *Streptococcus* Rosenbach 1884 emended Winslow and Rogers 1905

Synonyms: Sphaerococcus Marpmann 1885, not *Sphaerococcus* Agardh 1821; *Perroncitoa* Trevisan 1889; *Babesia?* Trevisan 1889; *Schuetzia* Trevisan 1889; *Lactococcus* Beijerinck 1901; *Hypnococcus* Bettencourt et al. 1904; *Myxokokkus* Gonnermann 1907, not *Myxococcus* Thaxter 1892; *Melococcus?* Amiradzibi 1907; *Diplostreptococcus* Lingelsheim 1912.

Chiefly parasites. Cells normally in short or long chains (under unfavorable conditions, sometimes in pairs and small groups, never in large packets). Generally stain by Gram. Capsules and zooglea often formed. On agar streak, effused translucent growth, often with isolated colonies. In stab culture, little surface growth. Sugars fermented with formation of large amount of acid. Generally fail to liquefy gelatin or reduce nitrates.

Type species is *Streptococcus pyogenes* Rosenbach.

3. *Staphylococcus* Rosenbach 1884

Synonyms: Micrococcus Cohn 1872 em. Migula 1894; *Botryomyces* Bollinger 1888; *Botryococcus* Kitt 1888, not *Botryococcus* Kuetzing 1849; *Galactococcus* Guillebeau; *Bollingera* Trevisan 1889; *Gaffkya* Trevisan 1885; *Pyococcus* Ludwig 1892; *Carphococcus* Hohl 1902; *Aurococcus* Winslow and Rogers 1906, *Indolococcus* Jensen 1909; *Liquidococcus* Jensen 1909; *Peptonococcus* Jensen 1909; *Enterococcus?* (Thiercelin) Rougentzoff 1914.

Parasites. Cells in groups and short chains, very rarely in packets. Generally stain by Gram. On agar streak good growth, of orange color. Sugars fermented with formation of moderate amount of acid. Gelatin often liquefied very actively.

Type species is *Staphylococcus aureus* Rosenbach.

4. *Albococcus* Winslow and Rogers 1905

Differs from *Staphylococcus* in forming more abundant surface growth of porcelain white color, and in fact that liquefaction of gelatin when present is less vigorous.

Tribe 2. Micrococceae Trevisan

Facultative parasites or saprophytes. Thrive best under aerobic conditions. Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cell aggregates in groups, packets or zooglea masses. Generally decolorize by Gram. Pigment yellow or red.

5. *Micrococcus* Cohn 1872, emended Winslow and Rogers 1905

Synonyms: Microsphaera Cohn 1872, not *Microsphaera* Leveille 1851; *Pediococcus* Balcke 1884; *Merista* Van Tieghem 1884, not *Merista* (Banks and Soland) Cunningham 1839; *Planococcus* Migula 1894; *Urococcus* Miquel 1879, not *Urococcus* Kuetzing 1849; *Pedioplana* Wolff 1907; *Tetradiplococcus?* Bartoszewicz and Schwarzwasser 1908; *Solidococcus* Jensen 1909; *Planomerista* Vuillemin 1913.

Facultative parasites or saprophytes. Cells in plates or irregular masses (never in long chains or packets). Generally decolorize by Gram. Growth on agar abundant, with formation of yellow pigment. Glucose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied, but not rapidly.

The type species is *Micrococcus luteus* (Schroeter) Cohn.

6. *Sarcina* Goodsir 1842, emended Winslow and Rogers 1905

Synonyms: Urosarcina Miquel 1879; *Planosarcina* Migula 1894; *Lactosarcina* Beijerinck 1908; *Sporosarcina?* Jensen 1909.

Sarcina differs from *Micrococcus* solely in fact that cell division occurs under favorable conditions in three planes, forming regular packets.

The type species is *Sarcina ventriculi* Goodsir.

7. *Rhodococcus* Flügge. 1891, emended Winslow and Rogers 1906

Synonyms: Not *Rhodococcus* Molisch 1907.

Saprophytes. Cells in groups or regular packets. Generally decolorize by Gram. Growth on agar abundant with formation of red pigment. Glucose broth slightly acid, lactose broth neutral. Gelatin rarely liquefied. Nitrates generally reduced to nitrites.

FAMILY VI. BACTERIACEAE Cohn 1872 emended

Rod-shaped cells without endospores. Gram-negative. Flagella when present peritrichic. Metabolism complex, amino-acids being utilized, and generally carbohydrates.

1. *Bacterium* Ehrenberg 1838, emended Jensen 1909

Synonyms: Actinobacter Duclaux 1882 in part; *Klebsiella* Trevisan 1885 in part; *Gliscrobacterium* Malerba and Sanna Salaris 1888; *Aerobacter* Beijerinck 1900; *Salmonella* Lignières 1900; *Denitrobacterium* Jensen 1909.

Motile or non-motile rods, staining evenly. Easily cultivable. Animal pathogens or saprophytes. Often chromogenic. Many forms actively decompose carbohydrates.

The type species is *Bacterium coli* Escherich.

2. ***Erwinia*** nov. gen.

Plant pathogens. Growth usually whitish, often slimy. Indol generally not produced. Acid usually formed in certain carbohydrate media, but as a rule no gas.

3. *Pasteurella* Trevisan 1887

Synonyms: Octopsis? Trevisan 1885; *Coccobacillus* Gamaleia 1888, not *Coccobacillus* Leube 1885.

Short rods, single or rarely in chains, usually showing distinct polar staining. Non-motile. Gram-negative. Without spores.

Aerobic and facultative. Powers of carbohydrate fermentation slight; no gas produced. Gelatin not liquefied. Parasitic, frequently pathogenic, producing plague in man and hemorrhagic septicemia in the lower animals.

The type species is *Pasteurella cholerae-gallinarum* (Flügge) Trevisan.

4. ***Hemophilus*** gen. nov.

Synonyms: Pyobacillus? Koppanyi 1907; *Diplobacillus* Morax 1896, not *Diplobacillus* Weichselbaum 1887.

Minute rod-shaped cells, non-motile, without spores, strict parasites, growing best (or only) in the presence of hemoglobin, and in general requiring blood serum or ascitic fluid. Gram-negative.

The type species is ***Hemophilus influenzae*** (Pfeiffer).

FAMILY VII. LACTOBACILLACEAE. Fam. nov.

Rods, often long and slender, Gram-positive, non-motile, without endospores. Usually produce acid from carbohydrates, as a rule lactic. When gas is formed, it is CO_2 without H_2. The organisms are usually somewhat thermophilic. As a rule microaerophilic; surface growth on media poor.

1. *Lactobacillus* Beijerinck 1901

Synonyms: Dispora? Kern 1882; *Saccharobacillus?* van Laer 1889; *Streptobacillus* Rest and Khoury 1902; *Brachybacterium* Troili-Petersson 1903; *Caseobacterium* Jensen 1909.

Generic characters those of the family.

The type species is *Lactobacillus caucasicus* (Kern?) Beijerinck.

FAMILY VIII. BACILLACEAE

Rods producing endospores, usually Gram-positive. Flagella when present peritrichic. Actively decompose protein media through the agency of enzymes.

1. *Bacillus* Cohn 1872

Synonyms: Bactrella? Morren 1830; *Metallacter?* Perty 1852; *Bactridium* Davaine 1868 in part; *Urobacillus* Miquel 1879; *Pollendera* Trevisan 1884; *Zopfiella* Trevisan 1885; *Streptobacter* Schroeter 1886; *Cornilia* Trevisan 1889 in part; *Bacterium* Ehrenberg, emended Migula 1894 in part; *Bactridium* Fischer 1895, not *Bactridium* Wallroth 1832; *Bactrinium* Fischer 1895; *Bactrillum* Fischer 1895; *Endobacterium* Lehmann and Neumann 1896; *Astasia* Meyer 1898; *Fenobacter* Beijerinck 1900; *Bacterius* Kendall 1902 in part; *Aplanobacter* E. F. Smith 1905 in part; *Semiclostridium* Maassen 1905; *Plennobakterium* Gonnermann 1907; *Myxobacillus* Gonnermann 1907; *Thermobacillus* Jensen 1909; *Serratia* Vuillemin 1913 in part, not *Serratia* Bizio 1823.

Aerobic forms. Mostly saprophytes. Liquefy gelatin. Often occur in long threads and form rhizoid colonies. Form of rod usually not greatly changed at sporulation.

The type species is *Bacillus subtilis* Cohn.

2. *Clostridium* Prazmowski 1880

Synonyms: Amylobacter Trecul 1865; *Cornilia* Trevisan 1889 in part; *Granulobacter* Beijerinck 1893; *Clostrillum* Fischer 1895; *Clostrinium* Fischer 1895; *Paracloster* Fischer 1895; *Semiclostridium* Maassen 1905; *Botulobacillus* Jensen 1909; *Butyribacillus* Jensen 1909; *Cellulobacillus* Jensen 1909; *Putribacillus* Jensen 1909.

Anaerobes. Often parasitic. Rods frequently enlarged at sporulation, producing clostridium or plectridium forms.

The type species is *Clostridium butyricum* Prazmowski.

ORGANISMS INTERMEDIATE BETWEEN BACTERIA AND PROTOZOA

SPIROCHAETACEAE Swellengrebel 1907

Free living or parasitic spirilliform organisms with or without flagella, with undulating or rigid spiral twists. Reproduction by transverse division and by "coccoid bodies," the equivalent of spores.

Four genera are recognized as follows:

1. *Spirochaeta* Ehrenberg. Non-parasitic, with flexible undulating body and with or without flagelliform tapering ends. Common in sewage and foul waters.

The type species is *Spirochaeta plicatilis* Ehrenberg.

2. *Cristispira* Gross. Giant forms with undulating body and peculiar flattened ridge erroneously called an "undulating membrane" which runs the length of the body. Parasitic in molluscs.

The type species is *Cristispira balbianii* Certes, from the crystalline style of the oyster.

3. *Saprospira* Gross. Non-parasitic forms similar to Cristispira, but without the flattened ridge or "crista" which is, if present, here replaced by a straight columella or thickening of the periplast.

The type species is *Saprospira grandis* Gross.

4. *Treponema* Schaudinn. Parasitic and frequently pathogenic forms with undulating or rigid spirilliform body. Without crista or columella. With or without flagelliform tapering ends.

The type species is *Treponema pallidum* Schaudinn.

1. ARTIFICIAL KEY TO THE ORDERS OF THE SCHIZOMYCETES

Cells united during the vegetative stage into a pseudoplasmodium
A. Myxobacteriales

Cells not forming a pseudoplasmodium
- Cells free or united in elongated filaments, often with a well-defined sheath. Conidia frequently formed. Free sulphur, iron or bacbacterio-purpurin often present.
  - Cells typically containing granules of sulphur or bacterio-purpurin or both..B. Thiobacteriales
  - Sulphur and bacterio-purpurin absent; iron often present
C. Chlamydobacteriales
- Cells never in sheathed filaments. Conidia only in the mycelial Mycobacteriaceae. Flagella often present. Free iron, sulphur, or bacterio-purpurin never present.........................D. Eubacteriales

2. ARTIFICIAL KEY TO THE FAMILIES OF THE EUBACTERIALES

Cells spiral with polar flagella..............................IV. SPIRILLACEAE

Not as above
- Cells spherical; rarely, if ever, motile; spores never produced; never securing growth energy from nitrogen or ammonia.........V. COCCACEAE

Not as above
Cells short rod-shaped with a single rarely two polar flagellum; usually forming green or yellow pigment..........III. PSEUDOMONADACEAE
Not wholly as above
Spores formed....................................VIII. BACILLACEAE
Spores never formed
Metabolism simple, securing growth energy from carbon, hydrogen, or their simple compounds; flagella, if present, polar
I. NITROBACTERIACEAE
Metabolism complex, dependent upon more complex carbohydrate and protein substances; flagella, if present, peritrichic
Cells clubbed, fusiform, filamentous, branching or mycelial; those not distinctly so are either acid-fast or show barred irregular staining........................II. MYCOBACTERIACEAE
Not as above
Gram-positive; non-motile...............VII. LACTOBACILLACEAE
Gram-negative; often motile..................VI. BACTERIACEAE

3. ARTIFICIAL KEY TO THE GENERA OF THE EUBACTERIALES

I. NITROBACTERIACEAE
Fixing nitrogen or oxidizing its compounds
Fixing nitrogen
Cells large; in soil....................................7. *Azotobacter*
Rods minute; in roots of leguminous plants............8. *Rhizobium*
Oxidizing nitrogen compounds
Oxidizing ammonia................................5. *Nitrosomonas*
Oxidizing nitrites...................................6. *Nitrobacter*
Not as above
Oxidizing hydrogen..............................1. *Hydrogenomonas*
Oxidizing carbon compounds
Oxidizing alcohol; branching forms common...........4. *Mycoderma*
Not as above, using simpler carbon compounds
Oxidizing CO................................3. *Carboxydomonas*
Oxidizing CH_4................................2. *Methanomonas*

II. MYCOBACTERIACEAE
Slender rods, staining with difficulty and acid-fast....3. *Mycobacterium*
Not as above
Mycelium and conidia formed
With aerial hyphae and conidia; usually saprophytic soil organisms..2. *Nocardia*
Hyphae and conidia not aerial; usually parasitic in animals
1. *Actinomyces*
Not as above; cells rod-like, usually somewhat curved, clubbed, fusiform, or even branched, but never mycelial
Thick, long threads, fragmenting into short thick rods
6. *Leptotrichia*

Not as above
Cells usually elongate and fusiform; filaments, if formed, not branching; staining somewhat irregularly.....5. *Fusiformis*
Cells slightly curved, clubbed, or in old cultures even branching; not filamentous; showing definitely barred staining....................................4. *Corynebacterium*

III. PSEUDOMONADACEAE
Generic characters mainly those of family..............1. *Pseudomonas*

IV. SPIRILLACEAE
Flagellum single (rarely 2 or 3)..............................1. *Vibrio*
Flagella tufted (5–20)....................................2. *Spirillum*

V. COCCACEAE
Abundant red-pigmented growth on agar................7. *Rhodococcus*
Not as above
Gram-negative
Normally in pairs of flattened cells; growth on plain agar scanty, never bright yellow..........................1. *Neisseria*
Normally in plates, packets, or irregular masses; growth on plain agar abundant, pigment definitely yellow
Cells in regular packets...............................6. *Sarcina*
Cells not in regular packets.......................5. *Micrococcus*
Gram-positive (Exceptions rare and not easily confused with above genera)
Cells normally in chains, sometimes in pairs (especially in acid environment) never in large irregular masses. Gelatine rarely liquefied. Growth on plain agar usually translucent, never heavy, never yellow or orange......2. *Streptococcus*
Cells normally in groups or masses; (occasionally in plates in Albococcus?) chains short and irregular, if present. Gelatine often liquefied. Agar growth abundant, white to orange
Pigment orange (rarely lacking); gelatine often liquefied actively..................................3. *Staphylococcus*
Whitish to porcelain white; liquefaction less vigorous
4. *Albococcus*

VI. BACTERIACEAE
Plant pathogens..2. *Erwinia*
Not as above; saprophytes or in animal habitats (intestines, tissues, etc.)
Usually motile and exhibiting active fermentative powers; typically parasitic in intestines of man and higher animals; growing well on ordinary media......................1. *Bacterium*
Not wholly as above
Growing only in presence of hemoglobin, ascitic fluid or serum
4. *Hemophilus*
Growth on media scanty, but less sensitive than the above; short rods with tendency to bipolar stain..........3. *Pasteurella*

VII. LACTOBACILLACEAE
Generic characters mainly those of family............1. *Lactobacillus*

VIII. BACILLACEAE
Aerobic, usually saprophytic; cells not greatly enlarged (if at all) at sporulation...1. *Bacillus*
Anaerobic, often saprophytic; cells frequently enlarged at sporulation...2. *Clostridium*

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COUNTING THE LIVING BACTERIA IN MILK—A PRACTICAL TEST

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The method under test, in the series of analyses reported here, has already been described (1915, 1916a). A comparison of results obtained in a series of milk analyses by this and the standard plate method has also appeared (1916 b). The following advantages have been claimed for the method:

1. It is rapid, requiring only about one-eighth of the time needed for the standard plate method.
2. The technic is simple and requires little glassware or culture medium.
3. It furnishes a means of keeping a permanent record of each analysis.
4. Only the individual bacteria, or groups of the same, that are alive grow into colonies. This makes it possible to use the method for counting the bacteria in pasteurized milk, which is not possible by methods of direct microscopical examination such as those of Slack and Breed.

It seemed desirable to make further tests of the method, and if possible, under practical conditions, such as working in connection with the routine examination of a city milk supply. Through the kindness of Dr. M. J. Rosenau of the Department of Preventive Medicine and Hygiene of the Harvard Medical School, arrangements were made for me to carry on a series of tests in the laboratories of the Boston Board of Health. The work has been made possible further by the courtesy of Dr. F. H. Slack, director of the laboratories, and Dr. W. M. Campbell, who conducts the bacteriological work on milk.

In this laboratory all samples of milk collected by the milk

TABLE 1

The number of bacteria per cubic centimeters of milk obtained by different methods

DATE	NO.	KIND OF MILK	MICROSCOPICAL ESTIMATE (SLACK)	STANDARD PLATES				LITTLE PLATES (FROST)	RATIO
				Routine test		Duplicate (Frost)			
				Number of colonies	Bacteria per cubic centimeter	Number of colonies	Bacteria per cubic centimeter	Bacteria per cubic centimeter	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
I. March 15, 1916	(1) 1	Pasteurized	–	14–10	120,000			52,000	1 : 0.43
	(2) 2	Pasteurized		7–10	85,000			28,600	1 : 0.34
	(3) 3	Raw	+	253–228	2,500,000			1,560,000	1 : 0.62
	(4) 4	Pasteurized	–	23–29	200,000			197,600	1 : 0.98
	(5) 5	Pasteurized	–	9–17	130,000			31,200	1 : 0.24
	(7) 6	Pasteurized	–	13–15	140,000			54,000	1 : 0.38
	(8) 7	Pasteurized	–	24–25	245,000			218,400	1 : 0.88
	(9) 8	Pasteurized	–	116–155	1,400,000			1,040,000	1 : 0.74
	(10) 9	Pasteurized	++	70–31	500,000			1,560,000	1 : 3.1
	(11) 10	Pasteurized	+	237–204	2,000,000			1,690,000	1 : 0.84
	(12) 11	Pasteurized	+	223–193	2,000,000			854,000	1 : 0.43
II. March 16, 1916	(1) 12	Pasteurized		164–139	135,000			59,150	1 : 0.43
	(2) 13	Pasteurized	+	29–sp.	290,000			96,200	1 : 0.33
	(3) 14	Pasteurized	–	63–79	710,000			2,080,000	1 : 2.90
	(4) 15	Pasteurized	–	24–22	230,000			187,200	1 : 0.81
	(5) 16	Pasteurized	–	20–22	210,000			65,000	1 : 0.31
	(6) 17	Pasteurized	–	136–128	132,000			13,000	1 : 0.10
	(7) 18	Pasteurized	–	62–76	690,000			1,040,000	1 : 1.50
	(8) 19	Pasteurized	–	54–39	460,000			130,000	1 : 0.28
	(9) 20	Pasteurized	+	29–26	270,000			640,000	1 : 2.40
	(10) 21	Pasteurized	–	98–102	100,000			85,800	1 : 0.85
	(11) 22	Pasteurized	+	8–6	7,000,000			1,560,000	1 : 0.22
	(12) 23	Pasteurized	+	9–sp.	1,900,000			780,000	1 : 0.41

NOTE – = microscopic estimate below 500,000. + = estimate of 500,000 and over. Sp. = spreaders. Column 5 gives counts on duplicate plates.

III. March 17, 1916	(1)	24	Raw	−	Spreaders				182,000	
	(2)	25	Raw	−	Spreaders				132,000	
	(3)	26	Pasteurized	−	12–18	150,000			49,400	1 : 0.33
	(4)	27	Pasteurized?	+	68–74	700,000			1,040,000	1 : 1.46
	(5)	28	Pasteurized	−	20–10	150,000			7,800	1 : 0.05
	(6)	29	Pasteurized	−						
	(8)	30	Pasteurized	−	25–15	200,000			200,000	1 : 1
	(9)	31	Pasteurized	−	10–4	70,000			78,000	1 : 1.11
	(10)	32	Pasteurized?	−	4–16	100,000			260,000	1 : 2.60
	(11)	33	Pasteurized	−	34–48	500,000			936,000	1 : 1.87
	(12)	34	Pasteurized	−	12–4	80,000			78,000	1 : 0.97
IV. March 18, 1916	(1)	35	Raw	−	Spreaders				41,600	
	(2)	36	Raw	−	Spreaders				62,400	
	(3)	37	Raw	−	Spreaders				44,200	
	(4)	38	Raw	−	Spreaders				18,200	
	(5)	39	Pasteurized	−	8–0	40,000			3,120,000	1 : 78
	(6)	40	Pasteurized?	+	178–184	1,800,000			1,716,000	1 : 0.95
	(7)	41	Pasteurized	−	6–18	120,000			640,000	1 : 5.33
	(8)	42	Pasteurized	−	7–sp.	70,000			210,000	1 : 3.
	(9)	43	Pasteurized	−	10–18	140,000			1,040,000	1 : 7.43
	(10)	44	Pasteurized	+	133–127	1,200,000			13,780,000	1 : 11.50
	(11)	45	Raw	−	Spreaders				338,000	
				−	Spreaders				200,000	
V. March 20, 1916	(1)	46	Pasteurized	−	Spreaders				10,400	
	(2)	47	Pasteurized	−	Spreaders				18,200	
	(3)	48	Pasteurized	−	Spreaders				117,000	
	(4)	49	Pasteurized	−	Spreaders				832,000	
	(5)	50	Pasteurized	−	Spreaders				182,000	
	(6)	51	Pasteurized	−	Spreaders				8,800	
	(7)	52	Pasteurized	−	Spreaders					
	(8)	53	Pasteurized	−	6–2	40,000			1,157,000	1 : 29
	(9)	54	Pasteurized	+	64–80	700,000			1,339,000	1 : 1.91
	(10)	55		+	Spreaders					
	(11)	56		+	338–363	3,500,000			1,300,000	1 : 0.37
	(12)	57		−	8–6	70,000			43,900	1 : 0.62

TABLE 1—*Continued.*

DATE	NO.		KIND OF MILK	MICROSCOPICAL ESTIMATE (SLACK)	STANDARD PLATES				LITTLE PLATES (FROST)	RATIO
					Routine test		Duplicate (Frost)			
					Number of colonies	Bacteria per cubic centimeter	Number of colonies	Bacteria per cubic centimeter	Bacteria per cubic centimeter	
(1)	(2)		(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
VI. March 21, 1916	(1)	58	Raw	—	13–14	120,000	3–2	30,000	33,800	1:0.25:0.28
	(2)	59	Raw	—	0–0	−10,000	1–2	10,000	5,200	1:1 :0.52
	(3)	60	Raw	—	10–6	80,000	11–12	110,000	20,800	1:1.25:0.25
	(4)	61	Raw	—	7–5	60,000	10–0	50,000	26,000	1:80 :0.45
	(5)	62	Raw	—	11–9	90,000	sp–5	50,000	39,000	1:0.55:0.43
	(6)	63	Raw	—	0–0	−10,000	6–sp	60,000	7,800	1:6 :0.78
	(7)	64	Raw	—	1–3	20,000	1–2	10,000	7,800	1:0.50:0.39
	(8)	65	Raw	—	0–0	−10,000	2–sp	20,000	3,250	1:2 :0.32
	(9)	66	Raw	—	0–0	−10,000	1–4	25,000	ap.13,000	1:2.5 :1.30
	(10)	67	Raw	—	5–3	40,000	5–2	35,000	11,830	1:0.9 :0.30
	(11)	68	Raw	—	5–1	30,000	2–2	20,000	27,400	1:0.66:0.90
	(12)	69	Raw	—	0–0	−10,000	13–3	80,000	22,750	1:8 :2.27
VII. March 22, 1916	(1)	70	Pasteurized	—	6–2	40,000	sp–5	50,000	26,000	1:1.25:0.65
	(2)	71	Pasteurized	—	10–16	130,000	15–14	150,000	114,000	1:1.15:0.80
	(3)	72	Pasteurized	—	37–41	400,000	36–sp	360,000	520,000	1:0.90:1.30
	(4)	73	Pasteurized	—	10–8	90,000	−5–7	50,000	41,600	1:0.55:0.45
	(5)	74	Pasteurized	—	3–15	90,000	37–28	320,000	267,000	1:3.55:3.00
	(6)	75	Pasteurized	—	14–16	150,000	10–13	120,000	197,000	1:0.80:1.31
	(7)	76	Pasteurized	—	25–3	140,000	22–sp	220,000	44,200	1:1.57:0.31
	(8)	77	Pasteurized	—	sp–3	30,000	3–3	30,000	15,000	1:1 :0.50
	(9)	78	Pasteurized	—	11–5	80,000	9–10	100,000	35,750	1:1.25:0.44
	(10)	79	Pasteurized	—	48–53	500,000	160–120	1,400,000	1,222,000	1:2.80:2.44
	(11)	80	Pasteurized	—	29–31	300,000	24–20	200,000	299,000	1:0.66:1.00

VIII. March 23, 1916	(1)	81	Raw	+	246–257	2,500,000	sp–340	3,400,000	1,500,000	1 : 1.36 : 0.60
	(2)	82	Pasteurized	−	10–12	110,000	1–2	10,000	260,000	1 : 0.09 : 2.30
	(3)	83	Pasteurized	−	37–43	400,000	27–sp	270,000	520,000	1 : 0.67 : 1.30
	(4)	84	Pasteurized	−	27–24	250,000	30–sp	300,000	20,000	
	(5)	85	Pasteurized	+	49–56	500,000	sp–8–8	80,000	1,000,000	1 : 0.14 : 2
	(6)	86	Pasteurized	−	11–sp	110,000	10–sp	100,000	104,000	1 : 0.90 : 0.95
	(7)	87	Pasteurized	−	10–2	60,000	11–sp	110,000	20,000	
	(8)	88	Pasteurized	−			10–11	100,000	100,000	1 : 1
	(9)	89	Pasteurized	−	10–20	150,000	11–25	180,000	30,000	
	(10)	90	Pasteurized	−	19–23	200,000	10–10	100,000	36,800	1 : 0.50 : 0.18
	(11)	91	Pasteurized	−	39–sp	400,000	54–45	500,000	653,000	1 : 1.25 : 1.63
	(12)	92	Cream	+	0–0		sp–0		no growth	
IX. March 24, 1916	(1)	93	Raw	+	1–0	60,000	1–0	10,000	26,000	1 : 0.16 : 0.43
	(2)	94	Raw	+	6–sp	60,000	0–3	15,000	12,000	1 : 0.25 : 0.20
	(3)	95	Raw	+	6–sp	60,000	sp–2	10,000	13,000	1 : 0.16 : 0.21
	(4)	96	Raw	+	sp–mold		33–36	350,000	91,000	
	(5)	97	Raw	+	0–0	−10,000	4–1	25,000	30,000	1 : 2.50 : 3.00
	(6)	98	Raw	+	74	750,000	40–20	300,000	300,000	1 : 0.40 : 0.40
	(7)	99	Raw	+	3–4	40,000	6–3	45,000	104,000	1 : 1.12 : 2.60
	(8)	100	Raw	+	8–sp	70,000	sp9–sp5	70,000	73,000	1 : 1.00 : 1.05
	(9)	101	Raw	+	0–0	−10,000	0–0sp	−10,000	very few	
	(10)	102	Raw	+	400–800	6,000,000	368–268	3,120,000	1,040,000	1 : 0.48 : 0.16
X. March 27, 1916	(21)	103	Pasteurized	−	184–sp	1,800,000	sp–sp		+1,000,000	
	(3)	104	Pasteurized	−	146–sp	1,460,000	520–sp	520,000	5,200,000	1 : 0.35 : 3.5
	(9)	105	Pasteurized	−	24–sp	250,000	9–4	650,000	26,000	1 : 2.60 : 0.1
	(20)	106	Pasteurized	+	24	24,000,000	sp-sp		2,600,000	
	(25)	107	Pasteurized	−	sp	500,000	sp-sp		3,000,000	
	(27)	118	Pasteurized	−	19	190,000	sp-sp			
	(28)	109		−	24	250,000	sp-sp		1,560,000	
	(29)	110		−	67	650,000	sp-sp		1,830,000	
	(30)	111		−	88	900,000	230–sp	2,300,000	3,120,000	1 : 2.5 : 3.4

inspector and sent into the laboratory are examined microscopically by the Slack method, and rated as above or below the legal limit of 500,000 bacteria per cubic centimeter. Those samples rated as likely to show more than the permissible number of bacteria are plated out, as also are all samples of pasteurized milk. The presence of streptococci and pus is noted. The microscopical estimate is used further as a guide to the dilution needed in plating. Ordinarily duplicate plates of only one dilution are made. This is usually 1: 10,000.

At the time the routine plates were poured I made little plates. From sample 59 to sample 111 (table 1) I also made standard plate cultures.

The medium used for the ordinary plates was made according to the standard methods. It had a reaction of +1.5 and contained one per cent of agar. Ten cubic centimeters were used for each plate. The Petri dishes had clay tops. The temperature of incubation was 37.5°, and the time forty-eight hours. The air of the incubator was saturated with moisture. The counting was done either with the naked eye or with the aid of a reading glass.

The little plates were made in the manner previously described (Frost, 1916 a) and incubated five to seven hours. They were then dried, stained and counted. The same agar was used as that supplied the laboratory. It would have been better, no doubt, to have had a medium containing more agar, since some difficulty was experienced from spreaders, and in highly contaminated milks the little colonies were not so well individualized as would have been the case with a stiffer agar.

The results obtained from these comparative tests are given in table 1. The kind of milk is indicated, i.e., whether raw or pasteurized. The microscopical estimate by the Slack method; the plate counts obtained in the routine analyses, together with the number of colonies actually seen on the plates; the plate counts obtained by the writer; the results obtained from the use of the little plates; and the ratio which the results obtained by the different methods of analysis bear to each other, are indicated in the different columns of the table.

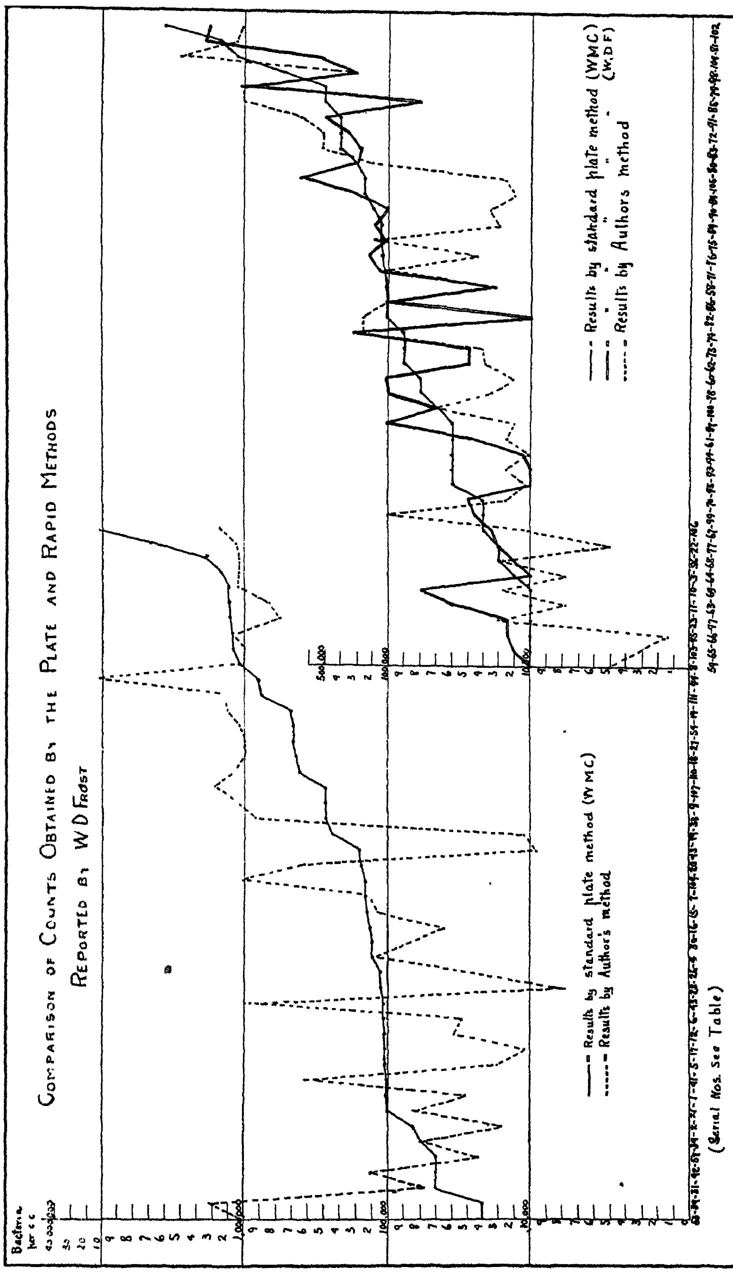
Comparison of Counts Obtained by the Plate and Rapid Methods
Reported by W D Frost
Results by standard plate method (WMC)
Results by Author's method
(Serial Nos. See Table)
Results by standard plate method (WMC)
" " " " (W.D.F)
Results by Authors method

CHART 1

All of the analyses made during the time of the comparative tests are included in the table, although it is apparent that the data are too unsatisfactory in a number of cases to be of much value; yet for the sake of completeness all are given.

The general results are shown in chart 1.

The milks used were both raw and pasteurized, and varied in bacterial content from very good to highly contaminated milks.

One of the first things to note is the fact that because of spreaders on both plates (which unfortunately were unusually prevalent in the laboratory at the time of these tests), no counts could be obtained in 16 out of the 111 tests, and in 16 other samples one of the duplicate plates was spoiled by spreaders.

While spreaders occurred on the little plates under exactly the same conditions, their presence did not materially interfere with a count of the plates, with the possible exception of one. It would seem then that the little plates could be relied upon to give countable colonies with more certainty than the standard plates.

The second thing to notice is that there is considerable variation between the counts on the duplicate plates of the routine series. In ten samples the difference amounted to over 50 per cent (5, 9, 26, 28, 30, 43, 71, 78, 89, and 102) and in eleven other samples it was over 100 per cent (31, 32, 34, 39, 41, 64, 68, 70, 74, 76, and 87).

Again, the number of colonies on the plates in about 40 cases was below 20, which would seem to be the smallest number of colonies that can be relied upon as giving anything like a satisfactory degree of accuracy.

Excluding those samples in which one-half or more of the plates were rendered useless because of spreaders, and also those samples in which both plates had less than twenty colonies, it will be seen that only 28 of the 111 counts can be considered as thoroughly reliable in indicating the exact number of bacteria in the sample when the standard plate method was used.

The criticisms here made of the routine plates would apply with equal force to the duplicate Petri dish cultures made by myself. Of 53 only 10 were entirely satisfactory in that both

plates were free from spreaders and had a sufficient number of colonies present to be thoroughly reliable.

The agreement between the two series of standard plates varies from a ratio of 1: 0.09 to 1: 8, with an average ratio of 1: 1.42. In other words, the plates that I made from one sample contained slightly less than one-tenth as many colonies (9 per cent) as the routine plates, and in another instance my count was 700 per cent higher than the routine, while my average figures were 42 per cent higher than those of Dr. Campbell.

The little plates, on the other hand, were all countable. Some of them were too thickly seeded, and others were overrun to some extent with spreaders, but neither of these things prevented the counting of the plates. It might have been expected, for several reasons, that the counts obtained by this method would not closely approximate the count obtained by the standard plate method, the chief of which is that the medium is somewhat different from the standard in that it is half milk. Again, the short period of incubation might not be sufficient for the development of colonies from some of the slowly-growing bacteria; and finally, crowding might be sufficient to inhibit the growth of some colonies. In spite of these differences and possible handicaps, however, the results obtained in this competitive series show a reasonable agreement.

Compared with the routine standard plates (leaving out of account three of the samples [39, 44 and 53] which are evidently not comparable), the ratio varied from 1: 0.05 to 1: 7.43, with an average of 1: 0.96. This appears to be a better showing than was obtained in duplicate runs by the standard plate method alone where the average variation was 1: 1.42. It must be pointed out, however, that this comparison gives a false impression, since the counts obtained by the little plates are on the whole somewhat lower than those obtained by the standard plate method. For example, if we compare the results I obtained by the standard plate method with the routine plates made by Dr. Campbell, we will see that my count fell below the routine count nineteen times, while it was higher in 14 samples. That is, the variation occurred either up or down with about

equal frequency. On the other hand, comparing the results obtained from the little plates with the results from the routine plates, it is found that in 48 samples the count on the little plates fell below the count obtained by the standard plate method, and above in 26 samples. In other words, the count on the little plates was lower than that on the standard plates in two cases, as against one above.

There are marked differences between the results obtained by the two methods in several samples. Some figures on the little plates are very much lower than those obtained by the standard plate method. This might be accounted for by the supposition that colonies did not have time to develop in the short period of incubation, but in some of these cases, at least, a long period of incubation had little effect in raising the count. What seems more likely in these instances is that we are dealing with samples of milks in which the bacteria have a tendency to form groups

Tests comparing the plating of 1/20 cc. of whole milk with the ordinary dilution methods. (By W. M. Campbell)*

1/20 CC. WHOLE MILK		DILUTION 1:20		DILUTION 1:100	
Number of colonies	Count	Number of colonies	Count	Number of colonies	Count
712	14,240	1,109	22,180	401	40,100
688	13,760	1,042	20,840	365	36,500
181	3,620	257	5,140	98	9,800
173	3,460	229	4,580	87	8,700
660	13,200	870	17,400	220	22,000
720	14,400	680	13,600	200	20,000
185	3,700	415	8,300	141	14,100
616	12,320	1,030	20,600	331	33,100
202	4,040	210	4,200	49	4,900
200	4,000	237	4,740	128	12,800
232	4,640	129	2,580	71	7,100
340	6,800			224	22,400
449	8,980			217	21,700
128	2,560	200	4,000		
110	2,200	240	4,800		
118	2,360	128	2,560		
96	1,920	140	2,800		

* American Journal Public Hygiene, 17, p. 359.

that are not easily shaken apart except as they are diluted with water. This fact has been brought out especially well by a series of tests given in the Reports of the Boston Board of Health (1907).

This grouping of the bacteria no doubt occurs to some extent in all milks, but appears to be more noticeable in the better grades of milk.

The little plates occasionally give much higher counts than the standard plate method; see, for example, nos. 39, 44, and 53 (table 1). In these samples the little plates showed a large number of small colonies of the lactic acid type, and it is quite conceivable that the count obtained by the little plates is more accurate than that obtained by the standard plate method.

TABLE 2

Number of bacteria in plain and lactose agars—from Sherman 1916

SAMPLE NUMBER*	NUMBER OF BACTERIA PER CUBIC CENTIMETER		
	Plain agar	Lactose agar	Ratio
1	817	1,017	1:1.25
2	1,340	2,070	1:1.55
3	1,630	2,000	1:1.23
4	3,230	3,530	1:1.09
(3)	6,000	69,000	1:10.50
(2)	6,500	42,500	1:6.54
5	7,330	7,330	1:1
(1)	7,600	25,300	1:3.33
6	8,000	11,300	1:1.41
7	8,900	12,100	1:1.36
8	9,030	8,970	1:0.99
9	11,800	11,170	1:0.95
(4)	18,100	43,600	1:2.41
(5)	23,800	58,000	1:2.44
(6)	72,000	177,000	1:2.46
10	186,000	610,000	1:3.28
11	192,000	278,000	1:1.45
12	260,000	361,000	1:1.39
13	369,000	463,000	1:1.26
14	53,700,000	109,000,000	1:2.03

* The numbers of the samples are the same as those used by Sherman in his table 1. The numbers in parenthesis are from his table 2. The last four analyses (nos. 15–18) are not given, because of the difficulty of charting such high numbers. See chart 2.

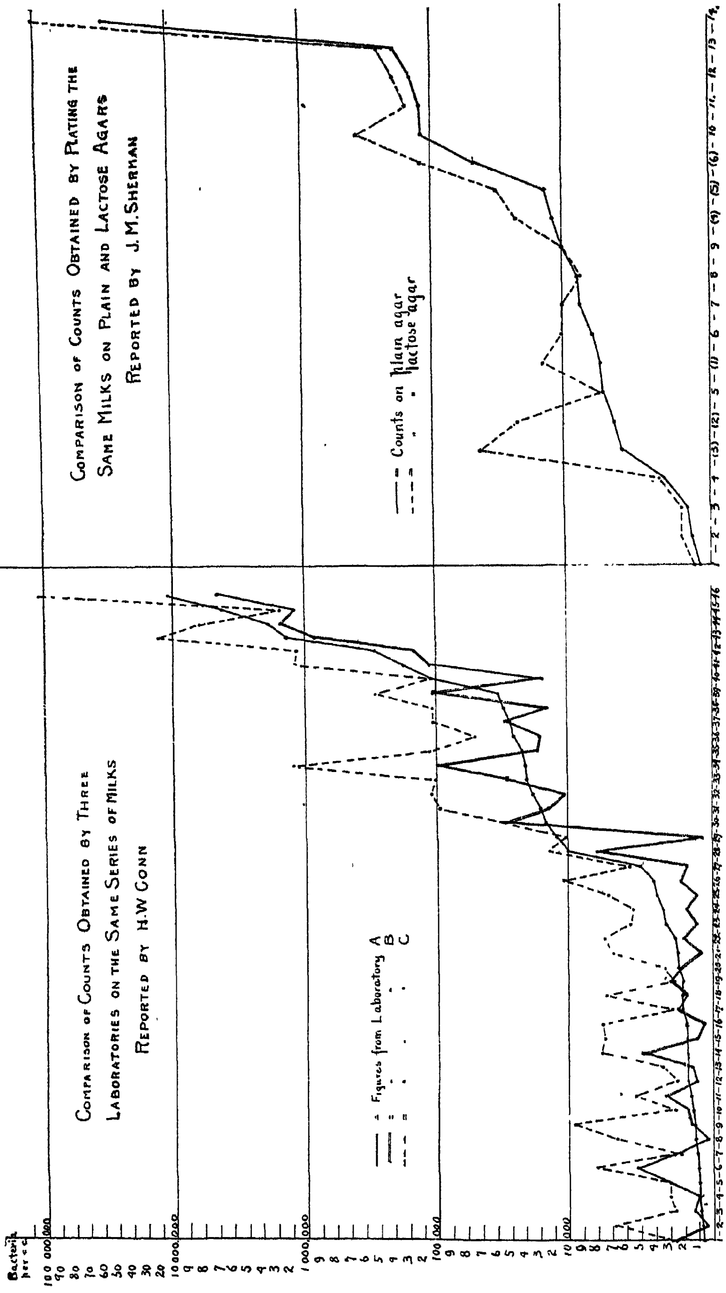
Comparison of Counts Obtained by Plating the Same Milks on Plain and Lactose Agars
Reported by J. M. Sherman
— = Counts on plain agar
- - - = " " lactose agar
Comparison of Counts Obtained by Three Laboratories on the Same Series of Milks
Reported by H. W. Conn
— = Figures from Laboratory A
═ = " " " B
- - - = " " " C
Bacteria per c.c.
100 000 000
10 000 000
1 000 000
100 000
10 000
(Serial Nos. see tables)

CHART 2

Sherman (1916) has recently shown that sugar agar gives higher counts than plain agar on the same milks. I have combined his tables 1 and 2 in my table 2. These figures are represented graphically in chart 2.

The large amount of milk in the little plates makes this a sugar medium, and it is possible that the discrepancies under discussion can be at least partially explained on this basis.

As far as the accuracy of the little plate method is concerned, when judged by the standard plate method, this study would seem to indicate that on the average the results are in fairly close agreement, with the probability that in about half of the samples the count will be somewhat low, and that other samples will show a much higher count than that obtained in the usual way.

It is interesting, in this connection, to compare the results obtained by different workers using the standard plate method in analyzing milk, and for this purpose I have brought together some of the results obtained by Conn (1915), and incorporated them in table 3. The figures were obtained by taking alternate numbers in Conn's table 2 and arranging them in an ascending scale. The column headed "S. Method and S. Media" was taken, but any other would apparently have given similar variations. The same figures are represented in chart 2.

TABLE 3

Data from "Standards for Determining the Purity of Milk". H.W. Conn. Table 3, p. 2366, Public Health Reports, August 13, 1915. Averages obtained by laboratories A. B. and C. Rearranged in one series using alternate figures.

RESULTS OBTAINED FROM THE SAME MILKS BY			RATIO
Laboratory A	Laboratory B	Laboratory C	
760	2,960	2,725	1:3.9:3.5
800	610	6,900	1:0.7:8.6
950	1,350	2,780	1:1.4:2.9
1,000	1,010	3,000	1:1.0:3.0
1,000	3,100	3,000	1:3.1:3.0
1,010	5,330	8,130	1:5.2:8.1
1,150	2,770	2,250	1:2.4:2.0
1,200	500	6,700	1:0.4:5.6
1,250	1,586	9,650	1:1.3:7.7
1,340	1,840	2,760	1:1.3:2.0

TABLE 3—*Continued*

RESULTS OBTAINED FROM THE SAME MILKS BY			RATIO
Laboratory A	Laboratory B	Laboratory C	
1,500	3,310	5,381	1:2.2:3.6
1,810	1,160	2,500	1:0.6:1.3
1,830	1,530	3,500	1:0.8:1.9
1,830	5,500	7,800	1:3.0:4.2
1,840	1,100	7,450	1:0.6:4.0
1,850	700	7,710	1:0.3:4.1
2,050	2,560	2,880	1:1.2:1.4
2,100	1,975	7,375	1:0.9:3.5
2,130	2,960	2,725	1:1.3:1.2
2,440	2,320	3,560	1:0.9:1.4
2,660	900	6,930	1:0.3:2.6
2,700	2,175	7,580	1:0.8:2.8
3,325	1,200	5,750	1:0.3:1.7
3,450	2,950	5,510	1:0.7:1.6
3,900	2,100	7,210	1:0.5:1.8
4,100	2,230	14,300	1:0.5:3.5
5,050	1,975	5,750	1:0.4:1.1
11,560	8,000	24,000	1:0.7:2.1
17,200	760	12,300	1:0.04:0.6
25,100	57,000	55,000	1:2.2:2.1
29,000	22,500	99,000	1:0.7:3.4
35,000	13,300	138,000	1:0.3:4.0
39,200	53,000	113,000	1:1.3:3.0
40,000	198,000	1,730,000	1:4.9:43.0
42,300	30,500	145,000	1:0.7:3.4
49,000	29,000	75,000	1:0.5:1.5
52,600	55,000	121,000	1:1.4:2.3
54,600	24,500	124,000	1:0.4:2.2
59,100	135,000	523,000	1:2.2:8.8
130,000	27,400	146,000	1:0.2:1.1
337,000	158,000	1,700,000	1:0.3:5.0
533,000	244,000	1,600,000	1:0.4:3.0
2,350,000	930,000	22,000,000	1:0.4:9.0
3,500,000	2,700,000	8,190,000	1:0.8:2.4
6,720,000	1,600,000	2,700,000	1:0.2:0.4
14,600,000	7,000,000	164,000,000	1:0.5:11.2

The variations, it will be noticed, are similar to those found in comparing my method with the standard plate method, and I believe I am warranted in saying that variations very similar in magnitude to those obtained by using the standard plate method and the little plate method may occur when two or more workers analyze milk by the standard plate method alone.

In considering the accuracy of this method, one of the things that must be determined is the uniformity of the distribution of the colonies on the little plates. Experience in these tests is that the distribution of colonies in the various fields in any little plate is quite uniform. To show this, the counts of twenty fields are given here by the mechanical selection of these fields on ten different plates.

NO. 1 LOW	NO. 5 LOW	NO. 10 OIL IM.	NO. 15 OIL IM.	NO. 20 LOW	NO. 30 LOW	NO. 40 OIL IM.	NO. 50 LOW	NO. 60 LOW	NO. 70 LOW
13	10	8	13	42	62	8	62	0	10
7	9	11	13	53	50	7	62	1	6
6	10	10	12	41	55	11	58	3	12
16	9	6	15	40	57	12	58	2	7
10	8	3	8	34	68	17	62	2	7
10	10	4	8	24	54	19	66	2	8
7	11	3	8	24	47	20	62	0	8
17	13	10	5	26	44	9	46	2	6
16	11	9	5	22	50	9	43	2	8
14	14	11	6	27	58	17	38	3	13
18	13	6	6	25	39	11	43	4	11
11	21	4	6	45	40	13	34	4	7
10	15	3	3	35	50	13	57	1	9
15	9	7	9	29	43	13	53	0	12
16	14	9	5	33	54	13	59	1	11
10	14	7	8	26	60	10	56	4	9
11	8	6	11	32	50	11	55	3	6
6	13	6	12	25	39	14	70	2	4
10	14	4	8	22	53	9	68	2	9
10	19	3	6	35	47	12	68	1	12
20)233	20)245	20)130	20)167	20)640	20)1020	20)248	20)1120	20)39	20)175
12	12	6.5	8	32	51	12	56	2	9

Another matter of importance is counting at different magnifications. In a former communication (1916 b) some emphasis

was laid on the discrepancies obtained with different magnifications used for counting the little plates. The data presented were for counts where the period of incubation was only four or five hours. If the period of incubation is seven or eight hours there seems to be little difference, and that magnification may be used which will give the most satisfactory number to count.

In comparing the two methods other considerations need brief mention.

First, the method is rapid, requiring not over seven or eight hours.

Second, the little plate method is reliable in that it is not likely to fail entirely because of spreaders, too great a dilution, etc.

Third, it permits a study of a relatively large amount of the milk, usually 1/20th of a cubic centimeter.

Fourth, it furnishes a permanent record of the bacterial content of the milk.

Fifth, it requires less material in the way of glassware and media. Microscopical slides, sterilized in the flame just before use, are substituted for Petri dishes, and the amount of medium is not over 1/20th and may be as little as 1/200th part of that used by the standard plate method.

Sixth, it is less time-consuming to make the little plates than it is to make the standard plates, and the only previous preparation necessary is the sterilization of pipettes and test tubes for mixing the samples. The counting of the little plates requires more time if 20 fields are carefully gone over. It seems probable, however, that the extensive counting may not be required in routine work. If the little plate is gone over quickly to gain an idea of the distribution of the colonies, the counting of four or five representative fields will be sufficient. Tests of this kind made during the progress of this work gave very satisfactory results. By varying the magnification, fields with comparatively few colonies in them can always be obtained.

The method may seem complicated to some, but in reality it is not, and if one will take the time to master the principles involved, the method is much simpler than the standard plate method.

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STUDIES ON THE BACTERIAL METABOLISM OF SULFUR

I. FORMATION OF HYDROGEN SULFIDE FROM CERTAIN SULFUR COMPOUNDS UNDER AEROBIC CONDITIONS

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Hydrogen sulfide is a decomposition product of many sulfur-containing compounds, and its formation by bacteria from both organic and inorganic substances has received much attention. An interesting paper on this subject is that of Sasaki and Otsuka, (1912). These investigators used Fränkel's protein-free medium as the substrate to which were added cystine, neutral sulfur, taurine, sodium thiosulfate, sodium sulfite, and sodium sulfate. All of the 21 pure cultures which they used formed hydrogen sulfide from cystine, with the exception of *Ps. pyocyaneus* and *Ps. fluorescens*. The pyogenic cocci were able to reduce neutral sulfur only. Burger (1914) observed hydrogen sulfide formation from cystine by a few common bacteria, but none of their strains produced it from taurine, peptone or sodium thiosulfate. Incubation periods of 12, 24 and 48 hours were used.

EXPERIMENTAL

In the course of some work that was being done with the fluorescent group of bacteria the action of these organisms, together with other strains of common bacteria, was tested upon cystine and other sulfur-containing compounds. The fluorescent bacteria used in this investigation were isolated from water. The other forms were secured from the American Museum of

Natural History, with the exception of the Bacillus of winter cholera.[1]

Fränkel's solution was used for the substrate. This was made up according to the following formula.

Sodium chloride	5 grams
Monocalcium phosphate	2 grams
Ammonium lactate	6 grams
Asparagin	4 grams
Distilled water	1000 cc.
$\frac{N}{1}$ NaOH	20 cc.

After the various sulfur compounds had been added the media were placed in test tubes and sterilized in the Arnold steam sterilizer. Inoculations were made with small portions of growth from a young slant agar culture.

To detect the formation of hydrogen sulfide, strips of bibulous paper which had been saturated with lead acetate solution containing glycerol were suspended in the top of each culture tube. The tubes were then plugged with cotton and incubated at 25°C. for thirty days, at the end of which time observations were made for darkening of the lead acetate paper. A full set of control tubes was employed. In no instance did the control tubes darken the indicator paper, which points to the fact that the hydrogen sulfide, when present in the culture tubes, came from the sulfur compounds in the substrate.

PEPTONE

Fränkel's medium with 4 per cent added peptone was used. Sixty-two of the 97 fluorescent bacteria formed hydrogen sulfide from peptone. Four of these strains produced it from peptone and not from cystine. This may be taken as evidence of other sulfur linkings in protein than that of cystine. Practically all of the pure cultures were able to split hydrogen sulfide from peptone.

[1] This organism was received from Mr. A. J. Hinkelmann, Galesburg, Illinois, and is described in the Illinois Medical Journal, November, 1915.

CYSTINE

The cystine used in this experiment was prepared from wool, according to Folin's method (Folin, 1910) and purified by repeated recrystallizations.[2] It was used in the strength of 6 per cent in Fränkel's medium. On account of the small supply of cystine, small culture tubes holding about 5 cc. were employed.

After incubation for thirty days 86 of the 97 strains of fluorescent bacteria had formed hydrogen sulfide from cystine. At the same time 34 strains of other common bacteria were inoculated into this medium, most of which formed hydrogen sulfide. The results are indicated in the table. *B. paratyphi "B," B. enteritidis*, the Bacillus of winter cholera, and *B. coli* formed large amounts of hydrogen sulfide from cystine in much less time than 30 days.

These data differ from those reported by Sasaki and Otsuka (1912). The fluorescent bacteria in their investigation formed no hydrogen sulfide. The discrepancy between the results of Sasaki and Otsuka and those reported in this paper may be due to the period of incubation. The former adopted one week as the incubation period, while in this investigation it seemed best to lengthen the period to 30 days. Many of the fluorescent bacteria isolated from water produced no hydrogen sulfide until after the fifteenth day. Most of the intestinal bacteria produced it in less than a week.

The decomposition of cystine has been studied from a chemical and biochemical viewpoint, but very few definite data are available. Mathews (1915) believes that in digestion in the intestine cystine is first formed, and that this later undergoes decarboxylation and deaminization to yield ethyl mercaptan, according to the following equation.

$$\begin{array}{ccccccccccccc}
 & H & & H & & H & & H & & H & & H & \\
 & | & & | & & | & & | & & | & & | & \\
H-S- & C & - & C & -COOH \rightarrow H-S- & C & - & C & -H+CO_2 \rightarrow H-S- & C & - & C & -H+NH_3 \\
 & | & & | & & | & & | & & | & & | & \\
 & H & & NH_2 & & H & & NH_2 & & H & & H &
\end{array}$$

[2] A portion of this was secured from Dr. H. B. Lewis of the division of physiological chemistry. The rest was prepared directly from wool.

The above changes are thought to be probable on account of the presence of mercaptans in the feces. Neuberg and Asher (1907), upon the dry distillation of cystine, obtained amino-ethyl-disulfide, $S_2(CH_2-CH_2-NH_2)_2$. It is improbable that this

TABLE 1

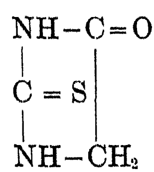
NAME OF BACTERIUM	NUMBER OF STRAINS	HYDROGEN SULFIDE FORMATION IN: Peptone +	Peptone −	Cystine +	Cystine −	2 thio-hydantoin +	2 thio-hydantoin −	Thio-urea +	Thio-urea −	Taurine +	Taurine −	$Na_2S_2O_3$ +	$Na_2S_2O_3$ −	$MgSO_4$ +	$MgSO_4$ −	Na_2SO_3 +	Na_2SO_3 −
Fluorescent bacteria	97	62	35	85	12		10*	62	35		97	42	55		97		97
B. coli	2	2	–	2	–	0	0	–	2	–	2	2	–	–	2	–	2
B. paracoli	1	1	–	1	–	0	0	1	–	–	1	1	–	–	1	–	1
B. enteritidis	1	1	–	1	–	–	1	1	–	–	1	1	–	–	1	–	1
B. alkaligines	1	1	–	1	–	0	0	1	–	–	1	–	1	–	1	–	1
B. cloacae	1	1	–	1	–	0	0	1	–	–	1	1	–	+	1	–	1
B. aerogenes	1	1	–	1	–	0	0	–	1	–	1	1	–	–	1	–	1
B. pyogenes-fecalis	1	1	–	1	–	0	0	–	1	–	1	1	–	–	1	–	1
B. paratyphi A	1	1	–	–	1	0	0	1	–	–	1	–	1	–	1	–	1
B. paratyphi B	1	1	–	1	–	–	1	–	1	–	1	1	–	–	1	–	1
B. typhi	1	1	–	1	–	0	0	–	1	–	1	–	1	–	1	–	1
B. capsulatus	3	3	–	3	–	0	0	1	2	–	1	3	–	–	3	–	3
Staph. albus	1	1	–	–	1	0	0	–	1	–	1	–	1	–	1	–	1
Staph. citreus	1	–	1	–	1	0	0	–	1	–	1	–	1	–	1	–	1
M. ureae	1	1	–	1	–	0	0	1	–	–	1	1	–	–	1	–	1
B. proteus	1	1	–	1	–	0	0	1	–	–	1	1	–	–	1	–	1
Clam bacillus	1	1	–	–	1	0	0	–	1	–	1	–	1	–	1	–	1
Sarcina ventriculi	1	–	1	–	1	0	0	–	1	–	1	–	1	–	1	–	1
B. butyricus	1	1	–	–	1	0	0	–	1	–	1	–	1	–	1	–	1
Bacillus of winter cholera	1	1	–	1	–	0	0	1	–	–	1	1	–	–	1	–	1
B. subtilis	1	–	1	–	1	0	0	–	1	–	1	–	1	–	1	–	1
Ps. pyocyaneus	1	–	1	+	–	0	0	–	1	–	1	–	1	–	1	–	1
M. tetragenus	1	–	1	–	1	0	0	1	–	–	1	–	1	–	1	–	1
B. cyaneus	1	1	–	–	1	0	0	–	1	–	1	–	1	–	1	–	1
Ps. phosphorescens	1	–	1	–	1	0	0	–	1	–	1	–	1	–	1	–	1
B. smegmae	1	1	–	–	1	0	0	–	1	–	1	–	1	–	1	–	1
B. arborescens	1	1	–	–	1	0	0	–	1	–	1	–	1	–	1	–	1
B. cereus	1	1	–	1	–	0	0	–	1	–	1	–	1	–	1	–	1
B. granulosus	1	–	1	–	1	0	0	1	–	–	1	–	1	–	1	–	1
B. mesentericus	1	1	–	1	–	0	0	1	–	–	1	1	–	–	1	–	1
M. flavus	1	–	1	–	1	0	0	–	1	–	1	–	1	–	1	–	1
B. dysenteriae	1	1	–	1	–	0	0	–	1	–	1	1	–	–	1	–	1

* Only the first ten strains were used in a 2 thiohydantoin medium.

compound would be formed in cellular metabolism, because the sulfur in cystine seems to be split off with little difficulty. Wohlgemuth (1905) reported that cystine which had been fed to rabbits in the diet appeared as increased sulfates in the urine and as taurine in the bile. Some of the hydrogen sulfide which many investigators have found in the urine may have come from cystine as well as sulfates.

The question of sulfur linkages in protein has received much attention. Johnson (1911) has given a good resumé of this subject. Many investigators have produced evidence which indicated that the sulfur in protein may not be fully accounted for by the cystine linkage. Bacteria may have a selective action, and may be able to attack only the sulfur in protein which is loosely combined. Johnson has prepared some thioamides of amino acids which offer more possibilities for the linkage of sulfur in protein.

2-THIOHYDANTOIN



In this compound sulfur has replaced the oxygen of the hydantoin nucleus. It has been studied by Johnson (1911, 1912) and his co-workers. Lewis (1912) has shown that the hydantoin nucleus is excreted as such when introduced into the organism of the cat, rabbit, or dog. In a later paper (Lewis, 1913) the same author reports a study of the behavior of 2-thiohydantoin when injected subcutaneously into rabbits. It was found "that approximately 0.125 gram per kilo body weight is the lethal dose for 2-thiohydantoin, while amounts of over 1.5 grams of hydantoin have been fed to rabbits without any toxic effects. The difference in reaction toward the animal body seems to rest in the sulfur of the 2-thiohydantoin.

Through the kindness of Dr. H. B. Lewis, a small amount of

2-thiohydantoin was secured upon which to try the action of bacteria. This was prepared from ammonium thiocyanate and hippuric acid, as described by Johnson and Nicolet (1911). The 2-thiohydantoin was dissolved in sterile water to yield a saturated solution. One cubic centimeter of this was added to 5 cc. of Fränkel's solution. The small amount of this compound limited the experiment to a few bacteria. The results are indicated in the table.

None of the bacteria formed the slightest amount of hydrogen sulfide detectable by the method used. The color of the solution changed upon incubation from the reddish orange of a solution of 2-thiohydantoin to a pale yellow. This may be regarded as indirect evidence that the compound was in some way broken down. Growth in all cases took place with the formation of a thick pellicle and a heavy sediment. This lack of toxicity for bacteria is of interest, in view of the marked toxicity for higher forms of life already referred to.

THIOUREA

A 10 per cent solution of this compound in Fränkel's medium was used. Thirty-four of the 97 fluorescent strains formed no hydrogen sulfide from this agent. Results with the other bacteria which were used may be found in the table. The formation of hydrogen sulfide from thiourea is of some interest, since none of the strains formed hydrogen sulfide from 2-thiohydantoin, a compound having the same sulfur linking, bivalent sulfur replacing bivalent oxygen. In peptone and thiourea substrates the fluorescent bacteria are correlated with regard to the number of strains forming hydrogen sulfide. This apparent correlation is probably of no significance. A strain which reduced the sulfur in peptone to hydrogen sulfide often failed to show this ability with regard to thiourea.

TAURINE

This was prepared from desiccated ox-bile and purified by repeated recrystallization (Hawk, 1916). None of the bacteria

used in this investigation reduced taurine to hydrogen sulfide, as determined by the method here employed. These results agree with those of Sasaki and Otsuka.

SODIUM THIOSULFATE

A 0.3 per cent solution of this compound in Fränkel's medium was used. Fifty-seven of the 97 fluorescent bacteria reduced the sulfur in sodium thiosulfate to hydrogen sulfide. The amount was not always large, but sufficient to cause a distinct change in the color of the lead acetate paper. The results for the other bacteria are indicated in the table. Lederer (1913) secured the formation of hydrogen sulfide when sodium thiosulfate was used in the place of sodium sulfate. Similar results have been reported by others.

MAGNESIUM SULFATE

To determine this property, Fränkel's solution was not used, but one proposed by Sullivan (1905) as being especially favorable to the development of fluorescent bacteria. It has the following composition.

Asparagin	10 grams
Magnesium sulfate	2 grams
K_2HPO_4	1 gram
Distilled water	1000 cc.

None of the bacteria reduced magnesium sulfate to hydrogen sulfide. This is in accord with the work of Sasaki and Otsuka and that of Lederer, and is probably due to the aerobic methods of culturing which were used. Beijerinck (1895, 1896, —) has shown that oxygen must be absent in order to secure formation of hydrogen sulfide from sulfate. An organism isolated by Zelinski (1893) seems to be the only one to which has been attributed the ability to form hydrogen sulfide from sulfate under both aerobic and anaerobic conditions.

SODIUM SULFITE

A 3 per cent solution of this compound was used in Fränkel's medium. None of the strains employed in this study reduced the

compound. This may have been due to the fact that very little growth was secured in the medium. Two of the strains, however, gave good growth, but no hydrogen sulfide. Sodium sulfite is known to inhibit the enzymes of the intestinal tract, and it may have acted in the same way with regard to the bacteria.

SUMMARY

The ability to form hydrogen sulfide from peptone is a rather wide-spread characteristic of the bacteria used in this study, including the fluorescent bacteria. With regard to cystine, the ability to liberate sulfur was possessed by almost all of the organisms used. The fluorescent bacteria and a few other forms are able to split hydrogen sulfide from thiourea with apparent ease, but do not attack 2-thiohydantoin to yield hydrogen sulfide. No hydrogen sulfide was formed from taurine and magnesium sulfate, both of which contain sulfur in more highly oxidized forms. The results with taurine and magnesium sulfate are in accord with those of Sasaki and Otsuka, indicating that bacteria may be unable to reduce highly oxidized sulfur with the formation of hydrogen sulfide. The results with sodium thiosulfate differ slightly from those reported by other investigators. With regard to sodium sulfite, no strain reduced sulfur to hydrogen sulfide, but this may be due to the concentration used. More work on this subject is planned.

I wish to acknowledge my indebtedness to Dr. Howard B. Lewis for his personal interest and timely suggestions.

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EARLY INSTRUCTORS IN BACTERIOLOGY IN THE UNITED STATES

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I have collected from various sources information concerning the early instructors in bacteriology in the United States. I have been especially fortunate in that it has been possible to obtain much of the information from the teachers themselves, though in many instances my informants were obliged to rely, in part, on their memory as to the dates when attention commenced to be directed to the bacteria and instruction was offered to students. I have attempted to arrange, in proper sequence, the order in which these several teachers began their work, but I may have failed to give the exact order. Other names might have been included in this list had it been possible to obtain the desired information. Following the publication by Pasteur of the results of his investigations on the relations of the bacteria to fermentation and to disease, several scientists in this country took up, independently, the study of bacteria by Pasteur's methods. These men were interested in the bacteria from either the broad biological standpoint or from the standpoint of pathology.

Probably the first name in the list of early teachers is that of the late Dr. T. J. Burrill who introduced the study of the bacteria into his course on the fungi during the "seventies." He discovered the organism of pear blight in 1879, and in the following years conducted, extensive inoculation experiments with this organism on a large orchard of young pear trees, thereby definitely establishing the etiological relation of the organism to the disease. In 1882 Dr. Burrill published a pamphlet entitled "Bacteria," in which he gives a discussion of the morphology, functions, classification and methods of study—the

methods of study being those of the French school. The classification of the bacteria follows that of Ferdinand Cohn. There is also included a brief description of the bacteria that were then identified. It was not, however, until 1892 that a course in general bacteriology was organized at the University of Illinois aside from the work which had been given in the course on the fungi.

Probably the next in order of seniority is the late Surgeon General George M. Sternberg, whose investigations on the causation of yellow fever, malaria, syphilis and other diseases are well known, who discovered the pneumococcus in normal sputum, and laid the foundations for our knowledge of the value of a large number of chemical substances as disinfectants. Dr. Sternberg translated Magnin's work on the "Bacteria" in 1880 and issued an enlarged and revised edition in 1884. In 1892 he published his large Manual of Bacteriology, and in 1896 his Text Book of Bacteriology.

The next in order is probably Dr. William H. Welch who from 1878 on was interested in the bacteria and their relation to disease, demonstrating them in sections of tissues to his classes in pathology at the Bellevue Hospital Medical College; and after Koch's discovery of the tubercle bacillus the study of this organism in sputum and tissues became an important part of the instruction. On returning from Europe in 1885 Dr. Welch became the head of the Pathological Institute at Johns Hopkins University, and had for his assistant in the instruction in bacteriology Dr. A. C. Abbott who had been Dr. Sternberg's assistant in the Biological Laboratory the previous year. Later on Dr. George H. F. Nuttall also became his assistant, and was associated with Dr. Welch in the discovery and study of the "gas" bacillus.

About the same time that Sternberg and Welch were directing their attention to the bacteria, Dr. T. Mitchel Prudden at the College of Physicians and Surgeons in New York also taught the staining of bacteria in sections of tissues and in sputum to his students in pathology, and commenced the cultivation of bacteria on solid media about 1883. Dr. Prudden, aside from

his interest in the pathological action of bacteria, also very early interested himself in the relation of bacteria to air, water, and ice, which were subjected to critical study, the results forming the basis of valuable monographs.

Beginning about 1879, Dr. D. E. Salmon commenced his important studies on the relation of bacteria to animal diseases in the Bureau of Animal Industry at Washington, and while Dr. Salmon is not known generally as a teacher of bacteriology, there is every evidence that he was the instructor of assistants in the Bureau and was the leading inspiration for many of the early discoveries made by the Bureau staff; notably the epoch making work which he did in association with Dr. Theobald Smith on Texas cattle fever, work which in a broad sense can be included here, even though the organism responsible for the disease is not a bacterium, but a protozoon.

In the group of instructors in pathology in medical schools who were interested in the relation of the bacteria to disease was the late Dr. Henry Formad at the University of Pennsylvania. In the autumn of 1882, Dr. Formad, who had visited and studied in Koch's laboratory during the summer, taught in class the method of diagnosing tuberculosis by staining the bacilli in sputum, and demonstrated various bacteria and the methods of cultivating them. In the autumn of 1883, after another visit to Koch's laboratory, Dr. Formad gave more extended demonstrations to his class of the apparatus and methods that were then in use by Koch in his laboratory. Dr. Formad made an investigation into the cause of diphtheria in an outbreak occurring in the state of Michigan, being associated in this work with Dr. Horatio C. Wood. Unfortunately Dr. Formad's inadequate training led him to erroneous conclusions with regard to the cause of diphtheria. The diphtheria organism seems to have escaped him, and he attributed the disease to a micrococcus. My first introduction to the bacteria was obtained in Dr. Formad's laboratory and from his lectures on the germ theory of disease.

Dr. W. T. Councilman, though not a bacteriologist, belonged in that group of early teachers of pathology who demonstrated

the bacteria in tissues and in secretions as a part of the routine course in pathological histology, and in this connection he became the director of students who later devoted their lives to a study of the bacteria.

Dr. A. C. Abbott became Dr. Sternberg's assistant at Johns Hopkins University in 1884, and assisted in his extensive studies on the value of chemical disinfectants, thus receiving his first training in bacteriology from Dr. Sternberg. In the spring of 1885, when Dr. Welch was made the director of the Pathological Institute, Dr. Abbott became his assistant as instructor in bacteriology and commenced to use the improved methods which Dr. Welch had learned in Koch's laboratory. Dr. Abbott had as his first student, in the spring of 1885, Dr. W. D. Booker, of Baltimore, who conducted an important investigation on the "summer complaints" of children; and shortly afterwards he also had as his student Dr. George H. F. Nuttall, now of Cambridge, England. Dr. Welch and Dr. Abbott were the first in this country to confirm the etiological relation of the diphtheria organism to the disease diphtheria, which had been announced from the German laboratories. When the Laboratory of Hygiene, University of Pennsylvania, was opened in 1892 under the direction of Dr. John S. Billings, Dr. Abbott became the first assistant, and a course in bacteriology was commenced at once. This course has been given to graduates in medicine and to other qualified students by different assistants in the laboratory under the direction of Dr. Abbott, and has been continued to the present time. In addition to this, all the instruction in bacteriology to the medical students has been given by the laboratory staff since 1896, and for some years both dental and veterinary students also received their instruction in bacteriology in the Laboratory of Hygiene. In 1892 Dr. Abbott published his Principles of Bacteriology, and this popular and valuable text book is now in its ninth edition.

Dr. Hermann M. Biggs took charge of the Carnegie Laboratory, at that time attached to Bellevue Hospital Medical College, in March, 1885, and began instruction in bacteriology to a class of graduate and undergraduate medical students.

The first formal course in bacteriology at the University of Wisconsin was given by Dr. E. A. Birge in the year 1885–1886. Some bacteriological apparatus and supplies had been purchased by the University at the request of Dr. William Trelease, and it was this apparatus which was utilized by Dr. Birge in this early course of instruction. Although Dr. Birge has maintained his interest in general biology, he has inspired some of our noted bacteriologists who studied under him, notably, Dr. H. L. Russell.

Dr. T. M. Cheesman began the first systematic instruction in bacteriological technique in Dr. Prudden's laboratory in the winter of 1885–1886. In the autumn of 1887 in the then new buildings of the College of Physicians and Surgeons on Fifty-ninth Street, systematic courses in practical bacteriology were offered to graduates in medicine and other technically qualified students.

Dr. John E. Weeks, who had spent the winter of 1884–1885 in Berlin studying pathology and bacteriology, entered the Ophthalmic and Aural Institute, 46 East Twelfth Street, as an interne. Dr. Herman Knapp returned from Europe in May, 1885, bringing with him appliances for bacteriologic and laboratory work, and asked Dr. Weeks to give a course in bacteriology. About November 1, 1885, a course, consisting of twenty lessons, was commenced at the Institute. This course was given to graduates in medicine only, and was repeated throughout the winters of 1885–1886 and 1886–1887.

Dr. Harold C. Ernst gave a course of six lectures on bacteriology at the Harvard Medical School in the fall of 1885. This was introductory to the course in general pathology given by Dr. R. H. Fitz.

Dr. Theobald Smith began a short course of lectures on hygiene which was largely bacteriological in the Medical Department of what is now the George Washington University in 1886. In 1894 he began to give laboratory instruction in bacteriology.

Beginning with the year 1886, Dr. L. H. Pammel began to direct the attention of the students in the Veterinary School of the

Iowa State College of Agriculture to the bacteria as a part of the course in cryptogamic botany. In the spring of the year 1889, Dr. Pammel began a general course in bacteriology to the senior veterinary students. This course was also offered to students in home economics.

Dr. Bayard Holmes gave a course in bacteriology to a class of three students in the Chicago Medical College (now Northwestern Medical School) during the spring of 1888, and in the spring of 1889 he became professor of bacteriology in the Postgraduate Medical School of Chicago. In July, 1889, he became secretary of the College of Physicians and Surgeons in Chicago and professor of bacteriology and surgical pathology, and began a systematic course in bacteriology, consisting of lectures and laboratory exercises, with a class of sixty students.

Dr. Victor C. Vaughn and Dr. F. G. Novy gave their first formal course in bacteriology in the winter of 1889, in the newly established Hygienic Laboratory of the University of Michigan. The course was elective, and was taken by students from the Medical and Literary Departments.

Dr. H. W. Conn first became interested in bacteria after hearing Dr. Farlow lecture in Boston in the year 1880 on "Low Forms of Plant Life," and had his first introduction to the bacteria at Johns Hopkins University under Dr. Councilman in 1881. Dr. Conn commenced to teach bacteriology about the year 1889, and his interest, aside from the broader biological significance of the bacteria, has been directed principally to the activities of the bacteria of milk and soil in their bearing on agriculture.

Dr. W. T. Sedgwick began the first formal course in bacteriology at the Massachusetts Institute of Technology during the academic year, 1888–1889.

Dr. Joseph MacFarland was appointed lecturer in bacteriology in the Medical School of the University of Pennsylvania in 1892, and gave a course of lectures with some laboratory exercises to the second year medical students. The course consisted in making hay infusions and studying the organisms that grew in them. Bacteriological studies of saliva, urine and sputum

were also made. The students were taught the ordinary methods of cultivation and staining, special attention being given to the staining of the tubercle bacillus.

Dr. William H. Park became professor of bacteriology at the Bellevue Hospital Medical College in 1895, when the first real teaching of bacteriology, as such, was begun by him.

From these simple beginnings the teaching of bacteriology has come in a comparatively brief time to play a very important part in the scientific education of many persons, and bacteriology is today being taught in a large number of educational institutions in this country. Courses are given, not only in elementary bacteriology to general science students but to students in domestic science, agriculture, dairying, water and sewage purification, public hygiene and sanitation, medicine, dentistry, veterinary medicine, pharmacy, brewing and fermentation industries, food production and preservation and plant pathology.

STUDIES ON THE NOMENCLATURE AND CLASSIFICATION OF THE BACTERIA

IV. SUBGROUPS AND GENERA OF THE COCCACEAE

R. E. BUCHANAN

From the Bacteriological Laboratories, Iowa State College, Ames, Iowa

Family 1. **Coccaceae** Zopf, 1884, p. 45, emended Migula 1894

Synonyms:

Sphaerobacteria Cohn, 1872, p. 146
Coccaceen Zopf, 1884, p. 45
Coccogenae Trevisan, 1885, p. 92
Coccacei Schroeter, 1886, p. 143
Coccobacteria Schroeter, 1886, p. 143
Sphaerobacteries Maggi, 1886, p. 81
Kokkaceen Hueppe, 1886
Coccacees Mace, 1897, p. 334

Cells usually spherical, sometimes somewhat elongated just before division, or when occurring in pairs or groups cells may be somewhat flattened on sides in contact. Never containing granules of sulphur, nor with bacteriopurpurin. Many species develop a pigment. Some species are motile by means of flagella. Cells frequently remaining in groups after division, forming chains, regular packets or irregular masses. Spore production doubtful, having been reported for but one species. Pseudoplasmodium never produced. Growth energy not secured by the oxidation of ammonia or nitrites.

The following names have been applied to groups designated as subfamilies, tribes and subtribes of the Coccaceae.

Ascococceae De Toni and Trevisan, 1889, p. 1037
Eu-Ascococceae De Toni and Trevisan, 1889, p. 1037
Gaffkyeae De Toni and Trevisan, 1889, p. 1042

Micrococceae De Toni and Trevisan, 1889, p. 1067
Sarcineae Trevisan, 1879, p. 136
Mycococcaceae Hansgirg, 1888, p. 266
Eucoccaceae Hansgirg, 1888, p. 267
Cystococcaceae Hansgirg, 1888, p. 266
Streptococceae De Toni and Trevisan, 1889, p. 1051
Homococcaceae Fischer, 1897, p. 32
Allococcaceae Fischer, 1903, p. 59
Metacoccaceae Winslow and Rogers, 1905, p. 669
Paracoccaceae Winslow and Rogers, 1905, p. 669

It is evident that, while many generic names have been proposed for cocci, very few authors have defined subfamilies, tribes or subtribes. Apparently the first effort was that of De Toni and Trevisan (1889, p. 1035). The following key to their tribes and subtribes indicates their grouping.

It will be noted that the chief emphasis throughout the classification is placed upon the development of cysts and capsules and upon cell groupings.

Key to Tribes of Coccogenae. De Toni and Trevisan

A. Cocci associated in families in bladder-like, gelatinous masses or cysts. Tribe I. *Ascococceae*
 I. Cocci separated from each other in the mucus of the matrix.
  a. Cocci destitute of special cysts, in families, in a common cyst. Subtribe I. *Eu-Ascococceae*
  b. Cocci borne in special cysts. Common cysts absent. Subtribe II. *Gaffkyeae*
 II. Cocci loosely united in a mucous matrix. Cysts generally delicate at length breaking apart. No special cysts. Subtribe III. *Amoebobacterieae*
B. Cocci associated in families of one or many layers, surrounded by a more or less evident mucous matrix........................Tribe II. *Sarcineae*
C. Cocci in moniliform chains. Large arthrospores developing in the filaments or at the tips................................Tribe III. *Streptococceae*
D. Cocci not in capsular cysts or sheaths, nor in moniliform chains. Small endospores in the cells..............................Tribe IV. *Micrococceae*

The classification of Winslow and Rogers (1905) separates the *Coccaceae* into two subfamilies, *Metacoccaceae* and *Paracoccaceae*. The subfamily *Metacoccaceae* is defined to include

the genera *Micrococcus*, *Sarcina* and *Rhodococcus*. The description given (1905, p. 669) is as follows: "Facultative parasites or saprophytes. Thrive best under aërobic conditions. Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles; cells aggregated in groups, packets or zoogloea masses." In later descriptions (1906, p. 845, and 1908, p. 258) they have added "Generally decolorize by Gram. Pigment yellow or red."

The subfamily *Paracoccaceae* (1905, p. 669) is defined to contain the genera *Diplococcus*, *Ascococcus*, *Streptococcus*, *Albococcus* and *Aurococcus*. The description is: "Parasites (thriving only, or best, on or in the animal body), thrive well under anaërobic conditions. Many forms fail to grow on artificial media; none produce abundant surface growths. Planes of fission generally parallel, producing pairs, or short or long chains." Later (1906, p. 545, and 1908, p. 249) the diagnosis was amended by adding: "never packets. Generally stain by Gram. Produce acid in dextrose and lactose broth. Pigment, if any, white or orange."

The careful work of the Winslows indicates that they have probably secured a logical basis for separation of the genera of the *Coccaceae* into two groups. It is not quite so certain, however, that the genera which they include are all that may be recognized. Perhaps the genus *Siderocapsa* Molisch is sufficiently distinct from the other cocci to require a third group in which to place it.

As has been previously shown (Buchanan 1915, p. 530) the subfamily names *Metacoccaceae* and *Paracoccaceae* are invalid in form, and might well be replaced by the tribal names *Micrococceae* Trevisan and *Streptococceae* Trevisan, with an emended diagnosis.

The three tribes recognized may be differentiated by reference to the following key in which are noted some of the distinguishing characters.

Key to tribes of Coccaceae

I. Not epiphytes, not causing the deposition of iron upon capsules.
  A. Commonly parasitic, in some forms saprophytic, usually thriving well under anaërobic conditions, not commonly producing luxuriant growths on artificial media, many forms failing to grow except upon special media. Cells in pairs, chains or irregular masses, never regular packets. Gram-positive, with exception of a few strict parasites.' Acid usually formed in dextrose and lactose broth. Pigment white, orange or absent.
  Tribe I. *Streptococceae*
  B. Saprophytes, or facultative parasites. Usually grow best under aërobic conditions, producing abundant to luxuriant growth on artificial media. Planes of fission often at right angles: Cells aggregated in groups, packets or zoogloeal masses. Usually Gram-negative. Pigment as a rule yellow, red or orange.
  Tribe II. *Micrococceae*

II. Epiphytes, usually on leaves and stems of water plants. Iron oxid deposited upon the capsule..............................Tribe III. *Siderocapseae*

The following list of generic, subgeneric and pseudogeneric names have been used for various cocci.

Merismopedia Meyen, 1839, p. 67 in part
Sarcina Goodsir, 1842, p. 432
Microhaloa Kützing, 1842, p. 169
Sphaerococcus Kützing, 1843, p. 408
Botryococcus Kützing, 1849, p. 892
Micrococcus Cohn, 1872b, p. 151
Microsphaera Cohn, 1872a, p. 237
Diplococcus Weichselbaum, 1874, p. 5
Ascococcus Billroth, 1874, p. 13
Coccobacteria Billroth, 1874, p. 1
Coccoglia Billroth, 1874, p. 7
Coccos Billroth, 1874, p. 4
Diplococcos Billroth, 1874, p. 5
Gliacoccos Billroth, 1874, p. 5
Megacoccos Billroth, 1874, p. 16
Mesococcos Billroth, 1874, p. 6
Micrococcoglia Billroth, 1874, p. 14
Micrococcos Billroth, 1874, p. 6
Monococcos Billroth, 1874, p. 5

Petalococcos Billroth, 1874, p. 6
Streptococcos Billroth, 1874, p. 10
Streptomicrococcos Billroth, 1874, p. 11
Ascococcus Cohn, 1875, p. 154
Leuconostoc Van Tieghem, 1878, p. 198
Gonococcus Neisser, 1879, p. 497
Chlamydatomus Trevisan, 1879, p. 137
Urosarcina Miquel, 1879, p. 517
Merista Van Tieghem, 1884, p. 1114
Staphylococcus Rosenbach, 1884, p. 19
Streptococcus Rosenbach, 1884, p. 22
Gaffkya Trevisan, 1885, p. 105
Neisseria Trevisan, 1885, p. 105
Arthro-Streptokokkus Hueppe, 1886, p. 145
Askokokkus Hueppe, 1886, p. 145
Hyalococcus Schroeter, 1886, p. 152
Leucocystis Schroeter, 1886, p. 152
Botryomyces Bollinger, 1888, p. 177
Pediococcus Balcke, 1884, p. 183
Pseudodiplococcus Bonome, 1888 p. 321
Haematococcus Babes, 1889, p. 81
Babesia De Toni and Trevisan, 1889, p. 1054
Bollingera De Toni and Trevisan, 1889, p. 10[illegible]
Perroncitoa Trevisan, 1889, p. 29
Schuetzia Trevisan, 1889, p. 29
Diplokokkus Baumgarten, 1890, p. 127
Tetracoccus Billet, 1890, p. 24
Endostreptokokkus Hueppe, 1891, p. 33
Mikrokokkus Hueppe, 1891, p. 33
Pediokokkus Eisenberg, 1891, p. 25
Sphaerokokkus Eisenberg, 1891, p. 50
Rhodococcus Zopf, 1891, p. 28
Iodococcus Miller, 1892, p. 63
Jodococcus Miller, 1892, p. 63
Makrokokkus Miller, 1892, p. 73
Pyococcus Ludwig, 1892, p. 27
Eucoccus Migula, 1895, p. 16

Euplanococcus Migula, 1895, p. 19
Euplanosarcina Migula, 1895, p. 20
Eusarcina Migula, 1895, p. 18
Planococcus Migula, 1894, p. 236
Planosarcina Migula, 1894, p. 236
Lactococcus Beijerinck, 1901, p. 213
Carphococcus Hohl, 1902, p. 338
Karphococcus Hohl, 1902, p. 338
Pseudosarcine Maze, 1903, p. 887
Mesococcus Smith, 1905, p. 174
Monococcus Smith, 1905, p. 174
Pneumococcus Schmidlechner, 1905, p. 291
Albococcus Winslow and Rogers, 1906, p. 541
Aurococcus Winslow and Rogers, 1906, p. 540
Myxokokkus Gonnermann, 1907, p. 883
Pedioplana Wolff, 1907, p. 9
Lactosarcina Beijerinck, 1908, p. 359
Tetradiplococcus Bartoszewicz and Schwarzwasser, 1908, p. 614
Liquidococcus Jensen, 1909, p. 305
Siderocapsa Molisch, 1909, p. 29
Metacoccus Conn, 1909, p. 12
Solidococcus Jensen, 1909, p. 332.
Sporosarcina Jensen, 1909, p. 340
Diplostreptococcus Lingelsheim, 1912, p. 494
Planomerista Vuillemin, 1913, p. 525
Enterococcus (Thiercelin) Rougentzoff, 1914, p. 648
Macrococcus, *Coccus*, *Meningococcus*, *Kokkus*, *Galactococcus*, etc. of many authors.

Many of these names may be discarded at once as invalid and not entitled to generic recognition.

The following have been used only as casual designations and are not valid genera: *Gonococcus*, *Pyococcus*, *Meningococcus*, *Galactococcus*, *Enterococcus*.

The following are the names of growth forms and have never been used in a strict generic sense: *Ascococcos*, *Coccoglia*, *Coccos*, *Diplococcos*, *Gliacoccos*, *Megacoccos*, *Mesococcos*, *Micrococcoglia*,

Micrococcos, Monococcos, Petalococcos, Streptococcos, Streptomicrococcus, Arthro-Streptokokkus, Tetracoccus, Endostreptococcus, Mesococcus, Pseudosarcina, Monococcus, Metacoccus, Macrococcus.

The following are invalid, usually Germanic, spellings of Latin words: *Askokokkus, Diplokokkus, Mikrokokkus, Pediokokkus, Sphaerokokkus, Jodococcus, Makrokokkus, Karphococcus, Kokkus, Myxokokkus, Haematokokkus.*

The following are names of algal genera in which certain bacteria were earlier placed, and are not true bacterial genera: *Gonium, Merismopedia, Sphaerococcus, Microhaloa, Gloeosphaeria.*

The following are invalid because previously used as generic names for plants not bacteria: *Microsphaera, Merista, Haematococcus, Botryococcus, Urococcus.*

The following are subgeneric designations: *Eucoccus, Euplanococcus, Euplanosarcina, Eusarcina.*

The following names are entitled to serious consideration as possibly valid names for cocci; *Sarcina, Micrococcus, Diplococcus, Ascococcus, Leuconostoc, Chlamydatomus, Staphylococcus, Streptococcus, Gaffkya, Neisseria, Hyalococcus, Leucocystis, Botryomyces, Pediococcus, Pseudodiplococcus, Babesia, Bollingera, Perroncitoa, Schuetzia, Urosarcina, Rhodococcus, Iodococcus, Planococcus, Planosarcina, Lactococcus, Carphococcus, Pneumococcus, Albococcus, Aurococcus, Pedioplana, Lactosarcina, Tetradiplococcus, Liquidococcus, Solidococcus, Sporosarcina, Diplostreptococcus, Planomerista, Siderocapsa.*

Certain genera which have been proposed can scarcely be recognized at the present time, because of insufficient characterization by the authors. Such genera are:

Leucocystis, Chlamydatomus, Ascococcus, Babesia, Iodococcus, Haematococcus.

Tribe I. **Streptococceae** Trevisan, 1889, p. 1051, emended

Synonyms:

Eu-Ascococceae Trevisan, 1889, p. 1035, in part
Gaffkycae Trevisan, 1889, p. 1042, in part
Paracoccaceae Winslow and Rogers, 1905, p. 1051

Cells spherical when isolated. Usually parasitic, growing well in the absence of oxygen, particularly in the presence of carbohydrates, from which acid is developed. Gas rarely produced. Many forms require special media. Abundant surface growths are rarely developed. Planes of fission are commonly parallel, resulting in the formation of pairs, or of longer or shorter chains of cells; regular packets of cells never formed. With the exception of a few strict parasites, the cells are Gram-positive. Pigment white, orange or none.

In a previous article (Buchanan, 1915, p. 541) it was suggested that the genera in this group to be recognized are *Leuconostoc*, *Streptococcus*, *Neisseria* and *Staphylococcus*.

A study of the characteristics of the genus *Neisseria* has led to the conclusion that it should be divided to form the genera *Neisseria* and *Diplococcus*, the former to include Gram-negative organisms, the latter the Gram-positive.

The following key to the recognized genera gives the most important of the differential characters.

Key to the genera of the Streptococceae

A. Cells occurring normally in chains.
  I. Usually parasitic. Not forming zoogloeal masses in sugar solutions....................................Genus 1. *Streptococcus*
  II. Saprophytic, occurring in cane sugar solutions in zoogloeal masses. Genus 2. *Leuconostoc*

B. Cells not occurring usually or characteristically in chains.
  I. Parasitic. Cells in pairs, growth as a rule meagre. No pigment formed.
    a. Gram-positive..........................Genus 3. *Diplococcus*
    b. Gram-negative..........................Genus 4. *Neisseria*
  II. Cells in irregular groups, usually parasites, growth as a rule good, pigment usually orange or white.......Genus 5. *Staphylococcus*

Genus 1. **Streptococcus** Rosenbach, 1884, p. 22; emended, Winslow and Rogers, 1905, p. 669

Synonyms:

Sphaerococcus Marpmann, 1889, p. 121 not *Sphaerococcus* Agardh, 1821, p. 227

Arthrostreptokokkus Hueppe, 1886, p. 144.

Perroncitoa Trevisan, 1889, p. 1053

Babesia? Trevisan, 1889, p. 29
Schuetzia Trevisan, 1889, p. 1052
Lactococcus Beijerinck, 1901, p. 213
Hypnococcus Bettencourt et al., 1904, p. 55
Myxokokkus Gonnermann, 1907, p. 883 not *Myxococcus*, Thaxter, 1892, p. 403
Melococcus? Amiradzibi, 1907, p. 309
Diplostreptococcus Lingelsheim, 1912, p. 494

Usually parasitic, though able to maintain a saprophytic existence. Cells usually in chains of greater or less length, the elements of the chains sometimes arranged as diplococci, never in packets. As a rule Gram-positive. Growth on surface of usual laboratory media not luxuriant. Growth better in liquid media containing sugars, which are as a rule fermented with acid production, but rarely gas. Usually do not liquefy gelatin or reduce nitrates. No formation of zoogloeal masses in sugar solutions.

The type species is probably *Streptococcus pyogenes* Rosenbach.

Genus 2. **Leuconostoc** Van Tieghem, 1878, p. 198; emended (as *Ascococcus*), Winslow and Rogers, 1905, p. 669

Synonyms:

Ascococcus Cienkowski, 1878, p. 12
not *Ascococcus* Cohn, 1875, p. 154
Leucocystis? Schröter, 1886, p. 152

Saprophytic, usually growing in cane sugar solutions. Cells in chains or pairs. Forming large zoogloeal masses when grown in sugar solutions. Produces acid actively from carbohydrates. Some types at least are Gram-negative.

The type species is *Leuconostoc mesenteroides* (Cienkowski) Van Tieghem.

Genus 3. *Diplococcus* Weichselbaum, 1886, p. 506, emended

Synonyms:

Klebsiella Trevisan, 1885, p. 94 in part
Hyalococcus Schroeter, 1886, p. 152
Pseudodiplococcus Bonome, 1888, p. 321
Pneumococcus? Schmidlechner, 1905, p. 291

Parasites growing poorly or not at all on artificial media. Cells usually in pairs, capsulated. Fermentative powers high, most strains forming acid in dextrose, lactose, saccharose and inulin. The cells are Gram-positive.

The type species is *Diplococcus pneumoniae* Weichselbaum. It is possible that this genus should be combined with *Streptococcus*.

Genus 4. **Neisseria** Trevisan, 1885, p. 105

Synonyms:

Diplococcus Weichselbaum, in part

Parasites, growing poorly in most laboratory media. Cells usually in pairs, flattened, coffee-bean shaped. Gram-negative. As a rule showing considerable fermentative power in carbohydrate media.

The type species is *Neisseria gonorrhoeae* Trevisan.

Genus 5. **Staphylococcus** Rosenbach, 1884, p. 19

Synonyms:

Micrococcus Cohn, 1872 b, p. 151, in part
Botryomyces Bollinger, 1888, p. 177
Botryococcus Kitt, 1888, p. 247
not *Botryococcus* Kützing, 1849, p. 892
Bollingera Trevisan, 1889, p. 1039
Gaffkya Trevisan, 1885, p. 105
Pyococcus Ludwig, 1892, p. 27
Carphococcus Hohl, 1902, p. 338
Albococcus Winslow and Rogers, 1906, p. 541
Aurococcus Winslow and Rogers, 1906, p. 205
Liquidococcus Jensen, 1909, p. 332
Indolococcus Jensen, 1909, p. 340
Peptonococcus Jensen, 1909, p. 340
Enterococcus (Thiercelin?) Rougentzoff, 1914, p. 648

Usually parasitic, cells as a rule in irregular groups or short chains, rarely in true packets, usually Gram-positive. Growth fair to good on the surface of artificial media. Sugars as a rule

fermented with acid. Gelatin commonly liquefied. Nitrates may or may not be reduced. Pigment orange or white.

It is probable that the genus should be divided upon the basis of pigment production into two genera, *Staphylococcus* and *Albococcus*, or perhaps these should rank as subgenera.

The type species is *Staphylococcus aureus* Rosenbach.

Tribe II. **Micrococceae** Trevisan, 1889, p. 1067; emended (as *Metacoccaceae*), Winslow and Rogers, 1905, p. 669

Synonyms:

Sarcineae Trevisan, 1889, p. 1044, in part
Metacoccaceae Winslow and Rogers, 1905, p. 669

Saprophytes or facultative parasites. Usually grow best under aërobic conditions. Grow well on artificial media, producing abundant surface growths. Planes of fission often at right angles, cells aggregated in groups, packets or zoogloëa masses. Generally Gram-negative. Usually pigmented yellow, orange or red.

The Winslows have included three genera in this group, *Micrococcus*, *Sarcina* and *Rhodococcus*. A key for the quick separation of these genera may be constructed as follows:

Key to the genera of Micrococceae

A. Cells not in regular packets.
  1. Pigment generally yellow..................Genus 1. *Micrococcus*
  2. Pigment red...............................Genus 2. *Rhodococcus*
B. Cells in regular packets. Pigment yellow or orange......Genus 3. *Sarcina*

Genus 1. **Micrococcus** Cohn, 1872, p. 151, emended; Winslow and Rogers, 1905, p. 669

Synonyms:

Microsphaera Cohn, 1872a, p. 151
  not *Microsphaera* Leveille, 1851, p. 381
Ascococcus? Cohn, 1875, p. 154
Pediococcus Balke, 1884, p. 183
Merista Van Tieghem, 1884, p. 1114
  not *Merista* (Banks and Soland) Cunningham, 1839 p. 47
Planococcus Migula, 1894, p. 236

Carphococcus Hohl, 1902, p. 338
Urococcus Miquel, 1888, p. 518
not *Urococcus* Kützing, 1849, p. 206
Pedioplana Wolff, 1907, p. 91
Tetradiplococcus Bartoszewicz and Schwarzwasser, 1908, p. 614
Solidococcus Jensen, 1909, p. 332
Planomerista Vuillemin, 1913, p. 525

Saprophytes or facultative parasites. Cells in plates or in irregular masses (never in long chains or packets). Generally Gram-negative. Growth on agar abundant, with formation usually of yellow pigment. Dextrose broth as a rule slightly acid and lactrose broth neutral. Gelatin frequently liquefied.

The type species is *Micrococcus luteus* (Schroeter) Cohn.

Genus 2. **Rhodococcus** Zopf, 1891, p. 28, emended; Winslow and Rogers, 1906, p. 546

Synonyms:
Not *Rhodococcus* Molisch, 1907, p. 20

Saprophytes. Cells in groups or regular packets. Usually Gram-negative. Abundant growth with red pigment on surface of culture media. Slight acid from dextrose, none from lactose. Gelatin rarely liquefied. Nitrate usually reduced to nitrites, but not to ammonia.

The type species is probably *Rhodococcus roseus* (Flügge) Winslow.

Genus 3. **Sarcina** Goodsir, 1842, p. 432, emended; Winslow and Rogers, 1905, p. 659

Synonyms:
Lactosarcina Beijerinck, 1908, p. 359
Urosarcina Miquel, 1879, p. 517
Tetradiplococcus? Bartoszewicz and Schwarzwasser, 1908, p. 614
Planosarcina Migula, 1894, p. 236
Pseudosarcina? Maze, 1903, p. 887
Sporosarcina Jensen, 1909, p. 340

Saprophytes or facultative parasites. Division occurs under favorable conditions in three planes, producing regular packets. Usually Gram-negative. Growth on agar abundant with formation of yellow pigment. Dextrose broth slightly acid, lactose broth generally neutral. Gelatin frequently liquefied. Nitrates may or may not be reduced.

The type species is *Sarcina ventriculi* Goodsir.

Tribe III. **Siderocapseae** *Trib nov.*

Cells spherical or ovoid, non-motile, epiphytic upon the leaves and other parts of water plants. Have not been cultivated. Thick capsules enclosing the cells become encrusted with iron oxid.

One genus only has been described, *Siderocapsa.*

Genus 1. **Siderocapsa** Molisch, 1909, p. 29

One to many spherical to ovoid small cells embedded in a globule of capsular material, epiphytic on water plants. The species have not been cultivated. Best recognized by staining with Schiff's reagent. Motility not known.

The type species is *Siderocapsa Treubii* Molisch.

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BACTERIUM PYOGENES AND ITS RELATION TO SUPPURATIVE LESIONS IN ANIMALS

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Lucet (1893) published a preliminary note on the bacteriology of suppuration in cattle and gave a very brief description of a non-motile rod under the name of *Bacillus pyogenes bovis.* Grips (1898) described under the name of *Bacillus pyogenes suis* an organism frequently found by him associated with suppuration in swine. Künnemann (1903) used the name *Bacillus pyogenes bovis* for an organism found by him frequently in abscesses in cattle. Glage (1903) concluded that the two organisms described by Grips and by Künnemann were identical and proposed the name *Bacillus pyogenes,* or *Bacterium pyogenes,* according to the Migula classification. Probably the organism is identical with the polyarthritis bacillus described by Poels even earlier than the work of Grips. The organism has also been isolated from suppurative lesions in goats and sheep (Olt. 1908; Poels, 1910), and from a number of wild animals in zoölogical gardens.

The present writer has encountered the organism in the hog associated with polyarthritis, in abscesses in the lungs and elsewhere. Lesions of the uterus and udder of the cow have also been observed.

The fact that such a common pyogenic organism of the domesticated animals long escaped discovery by European workers, and still longer has escaped attention of workers in the United States, is a matter susceptible of explanation. It is very small and takes stains irregularly. On account of its pleomorphism, a microscopic examination of pus containing it would ordinarily lead to the conclusion that micrococci, rods, and possibly streptococci were present. However, its characteristic of failing

to grow well on common agar is by far the best explanation of why it escapes isolation. Even when serum agar or solid blood serum is used, cultures may not be obtained, if the amount used for seeding is small, and especially if the abscess is old, in which case most of the organisms have died.

ISOLATION IN PURE CULTURE

The present writer has had success in isolating the organism by the employment of serum agar plates as described by Künnemann (1903). Cattle serum is rendered sterile by filtration through a Berkefeld filter, and is distributed with aseptic precautions in small test tubes, each containing about 5 cc. of serum. Just previous to pouring the plates, the contents of one of these tubes is added to the cooled agar and thus constitutes about one-third of the bulk of the medium. Grips' method of streaking out the suspected material on slanted solidified blood serum also gives good results. Olt (1908) claims to be able by the use of a rabbit to isolate the organism when it is present in the suspected material in very small numbers. The material is injected under the fold of skin on the posterior border of the ear. Under these conditions, saprophytes die out rapidly, while *B. pyogenes* multiplies. After twenty-four to forty-eight hours the area is disinfected, a small amount of pus is squeezed out and cultures on slanted serum are made therefrom. He reports success in the use of the method on such material as scrapings from the soft palate of a cow, and the fluid that exudes on pressure from the tonsil of a pig.

Morphology. The organism is one of the smallest of the bacteria. It varies in size enormously under the different conditions afforded by exudates and the various culture media. The smallest form may appear as a mere visible point and is especially common in old abscesses. The rods vary from 0.3 to 2 microns in length, and are about 0.2 micron broad. In exudate forms resembling the Rotlauf bacillus are seen. Chain formation is not rare. Tapering forms may occur. Spore formation, capsule, pigment production, motility and acid-fast character are absent. Irregularity of staining with carbol fuchsin is

common, but bipolar forms do not occur. The organism is stained readily by the ordinary basic anilin dyes. When stained with alkaline methylene blue, banding is common. Occasionally a long form will be found which displays darker stained areas at the ends, suggestive of *B. diphtheriae*. The Neisser stain does not reveal granules as in *B. diphtheriae*, for the cells are uniformly stained brown. The organism is Gram-positive, a fact that is useful when a microscopic search is made for it in exudate. However, writers have warned that the stay in alcohol should not be too prolonged. Discrepancies among the opinions of the earlier writers as to whether the organism is Gram-positive or not have been harmonized by Olt (1908). He explains that organisms that have been long dead in old exudates are Gram-negative, while live organisms give a Gram-positive reaction. Holth has modified the Gram method for staining tissues as given on p. 626.

Temperature requirements. Berger (1907) sets the range of temperature at which *B. pyogenes* will grow at 24° to 40°, with 37° as the optimum. He finds the thermal death point to lie between 55° and 59°. Milk cultures will remain alive longest at room temperature. The present writer has experienced poor success in keeping cultures alive in the ice box for as long as ten weeks without transplanting, although Berger reports success up to three months.

Conditions for cultivation. The organism is very *dainty* with respect to the culture media upon which it will grow. Serum bouillon, raw serum agar, solidified serum and milk are the most satisfactory media. Solidified blood serum or milk is most valuable for stock cultures. Growth does not occur on potato. Growth on raw serum and common media, such as agar, bouillon and media containing bouillon without the addition of serum, is so slight as not to warrant their use. Growth occurs in gelatin, with liquefaction, but is necessarily slow. The organism grows equally well under aerobic and anaerobic conditions, and in hydrogen as well as in oxygen.

Serum agar. Growth on this medium is detected after thirty-six to forty-eight hours in the incubator as minute points, and

several more days must elapse before maximum growth is reached. European writers describe the sub-surface colonies as having minute thin projections, when observed under weak magnification, giving the colony a thorny appearance. The present writer has observed the condition in the very smallest colonies, but does not find it constant, as might be expected from the great variation of the raw serum content of the medium. Surface colonies rarely exceed 3 mm. in diameter, and are relatively scarce in any plate. With low magnification under the microscope these colonies appear coarsely granular in the center. The granulations are of finer structure peripherally, and near the border the structure appears more uniform. The border line is regular. In optical qualities the colonies are smoky brown by directly transmitted light and bluish white by reflected light.

The subsurface colonies do not frequently assume the ordinary lenticular form, but exhibit a wide variety of shapes, among which trefoil-like forms appear and colonies like the conventionalized heart are observed occasionally. Under low magnification of the microscope the borders of the colonies are rather sharp, but the surface is seen to be slightly irregular. The structure varies in appearance from finely granular with smooth edges, to more coarsely granular with correspondingly rougher edges. Older colonies are surrounded by halo-like areas of cloudiness.

Serum bouillon. The medium becomes clouded by finely flocculent grayish flakes that eventually become deposited as a sediment in the bottom of the tube and to some extent upon the sides, suggesting the behavior of a streptococcus culture. The supernatant fluid may be only slightly clouded or very much so, depending upon the percentage of serum present. The sediment consists of masses of agglutinative bacteria which, when shaken up, produce an irregular clouding of the fluid.

Berger has determined that the more serum the bouillon contains the better the development of the organism and the greater the amount of sediment. The evidence of agglutination in the fluid was found to be greatest when equal parts of serum and of bouillon were employed, and least when 1 part of serum was added to 10 parts of bouillon. He regards this latter propor-

tion as best for a medium to be used for both agglutination work and for animal inoculation.

Slanted, solid cattle serum. European writers describe evidence of growth occurring after several days in the form of pit-like depressions caused by liquefaction. In the experience of the present writer, the liquefaction is more likely to be general over the area seeded, and causes the formation of a trough-like or a shallow irregular depression in the surface of the serum resulting from the gravitation of the liquefied material. Concurrent with this the ordinary collection of fluid at the base of the slant increases in volume. This fluid may be clouded at first but eventually becomes clear with a whitish sediment. As liquefaction progresses the diameter of the mass of solid serum is diminished and the mass is surrounded by a clear fluid, sediment being deposited on the bottom of the tube. In the latest stages the mass of serum often cracks into pieces and eventually the greater part is transformed into clear fluid.

In the experience of the writer the organism frequently fails to grow freely upon solid serum, possibly due to evaporation taking place more rapidly than the liquefaction caused by the organism. The absence of liquefaction in cultures supposed to be *B. pyogenes* should not be given serious consideration until several trials have been made with freshly-prepared medium.

Milk. After forty-eight hours at 37° a slight amount of coagulation induced by development of acid is observed to have occurred in the bottom of the tube, and after three days the coagulation is complete. Separation of the whey occurs, followed by the liquefaction of more or less of the coagulum after a few days. Berger (1907) ascribes the action on the coagulum, as well as the changes induced in gelatin and solid blood serum, to the presence of a proteolytic ferment in the cultures. A culture killed at 59°, when added to solid serum, induced slight liquefaction.

Susceptibility of small animals. The organism has a low degree of pathogenicity for guinea-pigs and mice unless enormous quantities of material are injected. Rats and pigeons are said to be completely immune. The rabbit is available for experimental

inoculation. Subcutaneous injection quite often induces a local abscess. Intra-abdominal inoculation, depending upon the virulence of the organism and the dose, may cause an acute or a chronic peritonitis. The acute purulent peritonitis may cause death in from six to fourteen days, while the chronic form exhibiting adhesions and abscesses may not result fatally for weeks or months. Intravenous inoculation in the rabbit may give negative results or in some cause a pyemia with metastatic foci in various organs including the peritoneum and joints.

The rabbit inoculations made by the writer have resulted in only chronic infections, if any. A rabbit was injected intra-abdominally on November 19 with 3 cc. of a normal salt suspension of growth of *B. pyogenes* from solid serum. The animal died on April 1, autopsy revealing two pyogenes abscesses on the concave surface of the liver. One of these was about the size of a pea, and the other twice as large. The abscesses were united to the adjacent intestine by threads of connective tissue. *B. pyogenes* was isolated from the pus Another rabbit was inoculated intravenously on March 9 with a culture of *B. pyogenes* designated as No. 22. The animal died on September 2. The muscles of the left thigh had been replaced by masses of pus surrounded by very thin transparent walls. The femur had been broken in its lower third, but had reunited. The marrow was red and permeated with channels of pus throughout its whole length. The hip joint was surrounded by a mass of fibrous tissue. *B. pyogenes* was isolated in pure culture.

GENERAL CHARACTER OF LESIONS CAUSED BY BACTERIUM PYOGENES

Bacterium pyogenes differs from the common pyogenic organisms in that it possesses the peculiarity of stimulating the proliferation of connective tissue to form a tumor-like mass similar to granulation tissue, and of subsequently inducing in that tissue the necrotic changes leading to abscess formation. Thus, suppuration caused by *B. pyogenes* in this manner follows a slow chronic course.

In this peculiarity the development of the lesions of *B. pyogenes* bears a general resemblance to the changes occurring in actinomycosis and in tuberculosis. On account of this peculiarity of the organism and of the individuality of the lesions, the changes could more appropriately be classed as a specific infectious disease than among the wound infection suppurative processes. Olt (1904) has suggested the name pyemic cachexia, but pyobacillosis is more generally used.

The earliest stage in an inflammatory lesion is represented by a mass of hard, yellowish colored connective tissue possibly surrounded by a zone of grayish white connective tissue. Later purulent dissolution sets in at the center of the yellowish mass, which gradually becomes transformed into an abscess. Pyogenes pus is generally yellowish in color with a suggestion of green, pasty in consistency, and inodorous.

Holth (1907) has made a most thorough histological investigation of the development of these abscesses. The peripheral part of the small fibrous tumor consists of fibrous connective tissue with very numerous cells. The interior consists of large endothelial-like cells imbedded in a network of connective tissue fibers. These cells are considered as young connective tissue cells which appear weakly granular when stained with strong protoplasmic stains. The nucleus lies either in the center or in the periphery. Similar cells with two nuclei occur often, but multinuclear cells are rare. Among these cells lie imbedded round cells, singly or in groups. The greater proportion of both classes of cells exhibit necrotic changes. In the larger tubercles the isolated groups of round cells become larger and more numerous, especially in the center of the tumor, and here the disintegration of the cells appears. Sections of smaller nodules or of abscess walls, stained for bacteria, show that the larger connective tissue cells may contain enormous numbers of pyogenes bacilli, which are usually short and plump, but which often appear as granules. The round cells contain only a few bacilli. In the degenerated tissue the organisms lie free, the rod shape predominating.

Holth has suggested the following modification of the Gram staining method for use in demonstrating *B. pyogenes:*

The sections are treated one to two minutes with anilin gentian violet. One pours the stain off and puts on the Lügel's solution which is allowed to act one-quarter to one-half minute. Thereupon the sections are immersed in absolute alcohol in which they remain until completely dehydrated. The alcohol is removed with the help of xylol, and the sections then have a bluish black appearance. Then oil of cloves is allowed to act about a minute, after which differentiation is accomplished in a mixture of 1 part of absolute alcohol and 4 parts of xylol, especially to remove the adhering stain and oil of cloves. In closing, pure xylol and balsam are applied.

In this method the bacteria are stained bluish black and the tissue pale reddish color. In objects fixed in formalin the red corpuscles and the elastic tissue take the stain very strongly. They show then a more or less bluish appearance.

The organism is also capable of inducing pure exudative changes in tissue, in inflammations following a chronic course. The exudate is usually a thin slimy fluid, having a whitish yellow, gray, or reddish yellow color. It may be sero-purulent, hemorrhagic or purulent, and generally contains considerable fibrin.

The infection very frequently spreads by metastasis along the blood and lymph streams.

Lesions frequently are found to contain *B. pyogenes* alone, but a wide variety of other suppurative organisms may be associated with it.

A review of the literature of *B. pyogenes* from European sources leads to the conclusion that it plays a most important rôle in animal diseases. In this place I shall no more than attempt to point out the breadth of the field of its activities in animal pathology.

Abscess formation in swine induced by this organism may occur in the widest variety of locations, in the subcutis, the musculature and the skeleton. Likewise, the peritoneum and visceral organs are subject to attack. A somewhat extensive literature has grown up about its relation to lung lesions alone. Here its importance equals, if it does not exceed, that of the swine plague organism. Grips, Glage and Nieberle (1904) state that *B.*

pyogenes may be recognized in the lungs of every case of swine plague. Pütz (1904) found it in 55 per cent of all cases, and Holth (1907) is of the opinion that 55 per cent is too low. It is stated that in old pneumonias *B. suisepticus* may be found, but very frequently it may be shown that this organism is only slightly virulent for experimental animals. Therefore, such organisms are indistinguishable from strains morphologically and biologically identical, which frequently exist in the bronchial contents under normal conditions. *B. pyogenes* seems to be a normal inhabitant of the mouth and of the tonsilar crypts. The intestinal mucosa is often the site of purulent catarrh induced by this organism.

In cattle as in swine *B. pyogenes* causes suppuration in a great variety of locations. Künnemann (1903) has isolated it from 90 per cent of specimens of bovine abscess pus. In 35 per cent of cases he found the organism alone in pus, in 55 per cent in company with other bacteria. It was found associated with other organisms almost uniformly in metritis, mammitis and navel infection. Pneumonia similar in appearance to tubercular pneumonia is caused by *B. pyogenes* in cattle and has been studied by Berger (1908). Poels has made extensive studies of arthritis (1912) and mammitis (1910) caused by this organism.

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INFLUENCE OF HYDROGEN-ION CONCENTRATION OF MEDIUM ON THE REPRODUCTION OF ALFALFA BACTERIA

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The value of the hydrogen electrode in determining the reaction of culture media has been shown by Michaelis (1914), Clark (1915, 1917), and others. Their results indicate that bacterial processes are influenced to a marked degree by the hydrogen-ion concentration of the medium. It appears that certain species of organisms are very sensitive to slight changes, while others will develop in a medium having considerable variation in reaction.

The question arises whether the reaction of a medium within certain limits exerts an important influence on the growth of legume bacteria. In this laboratory an attempt has been made to study the effect of reaction on the growth of *B. radicicola* from alfalfa.

METHODS

Mannitol solution was used as a culture medium.

Mannitol	10.0 grams
Magnesium sulfate	0.2 gram
Monobasic potassium phosphate	0.2 gram
Sodium chloride	0.2 gram
Calcium sulphate	0.1 gram
Distilled water	1000.0 cc.

The phosphate was dissolved separately in a little water and the solution made neutral to phenolphthalein with N/1 sodium hydroxid. After sterilizing, the culture medium was again adjusted to the neutral point of phenolphthalein.

Hydrogen-ion concentrations were measured electrometrically. A modification of the apparatus used and described in detail by Loomis and Acree (1911) was used. All readings were made at 25°C. and are expressed in terms of the symbol P_H,

(Sørensen (1909)) representing the log I/C_H. Corrections were made for hydrogen pressure and for variations of the calomel electrodes, as suggested by Loomis and Acree (1916).

REACTION TO PHENOLPHTHALEIN AND HYDROGEN-ION CONCENTRATION

Twenty-four 100 cc. portions of mannitol solution in 750 cc. Erlenmeyer flasks were inoculated with alfalfa bacteria. The cultures were then arranged in pairs and the reaction changed by the addition of sterile N/10 sulphuric acid or sodium hydroxid. Column 2 of table 1 shows the variations in reaction. Immediately after inoculation the hydrogen-ion concentration was determined, and again after two weeks. (See chart 1.) In connection with the measurements of reaction, plate counts of the total number of bacteria were made after two and four weeks. The averages of triplicate plates, and the hydrogen-ion concentration, as well as the titrated acidity or alkalinity are given in table 1.

TABLE 1

Reaction and growth of legume bacteria

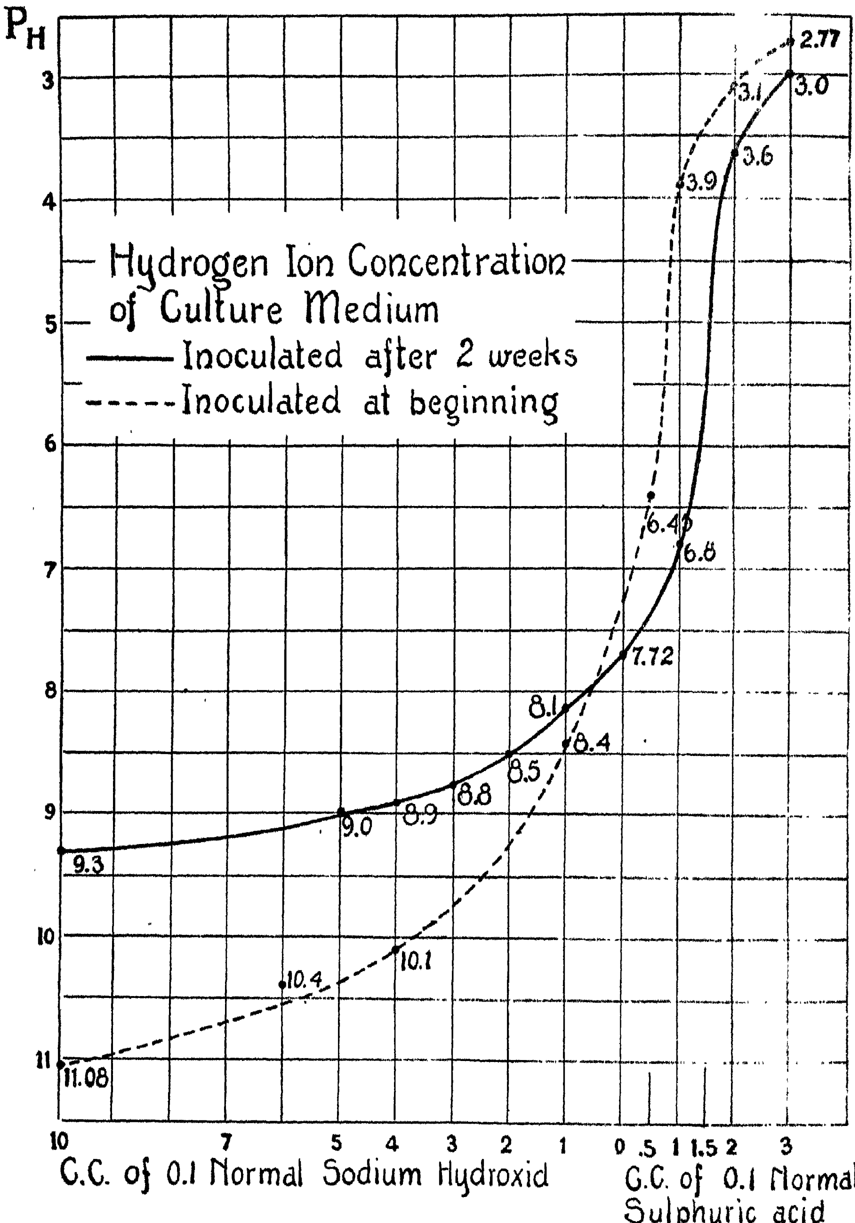
FLASK NUMBER	NORMAL ACID OR ALKALI IN 100 CC. OF MEDIUM	P_H			BACTERIA IN 1 CC. OF CULTURE MEDIUM			
		Beginning		After two weeks	After two weeks		After four weeks	
	Cubic centimeter	Theory in water	Found					
			In culture medium		Total	Relative	Total	Relative
1	0.3 H_2SO_4	2.52	2.77	3.00	No growth		No growth	
2	0.2 H_2SO_4	2.70	3.10	3.60	No growth		No growth	
3	0.1 H_2SO_4	3.00	3.90	6.80	19,580,000	75	31,000,000	58
4	0.05 H_2SO_4	3.30	6.45		21,520,000	83	32,500,000	61
5	Neutral	6.86	7.2	7.72	25,900,000	100	53,200,000	100
6	0.1 NaOH	10.72	8.4	8.1	20,280,000	78	29,200,000	55
7	0.2 NaOH	11.02		8.5	18,280,000	70	30,100,000	56
8	0.3 NaOH	11.20		8.8				
9	0.4 NaOH	11.32	10.1	8.9	13,220,000	51	29,800,000	56
10	0.5 NaOH	11.42		9.0				
11	0.7 NaOH	11.57	10.4		14,540,000	56	21,900,000	41
12	1.0 NaOH	11.72	11.1	9.3	12,100,000	46	11,600,000	21

The results show clearly the difference between the concentration of hydrogen ions and the total concentration of acid or alkali. From the hydrogen electrode measurements, it is evident that change in the hydrogen-ion concentration is much greater with a given increase in acidity than with a corresponding increase in akalinity, especially after bacterial growth has continued for two weeks. Consequently large amounts of alkali have little effect on the hydrogen-ion concentration of the mannitol medium, while even small amounts of acid cause a decided change in true acidity. The difference in the P_H for a given amount of acid and alkali in water solutions and in the mannitol medium is very noticeable from the figures of table 1. Here the vertical column 3 gives the theoretical P_H in water solutions and columns 4 and 5 the P_H found in the culture medium when inoculated. The buffer action may be due to the mannitol or to products of the bacterial growth. The latter seems more probable, because it is so much more noticeable after two weeks' growth.

HYDROGEN-ION CONCENTRATION AND GROWTH OF BACTERIA

The most important point shown by the data of table 1 is the relation between hydrogen-ion concentration and development of alfalfa bacteria. Plate counts after two and four weeks indicate that *B. radicicola* is much more sensitive to sulphuric acid in mannitol solution than to gram equivalent amounts of sodium hydroxid. Somewhat similar results have been reported by Prucha, (1915), who found that normal hydrochloric acid was much more injurious to the multiplication of *B. radicicola* of alfalfa than equivalent amounts of normal sodium hydroxid.

It was assumed in previous investigations that *B. radicicola* from alfalfa was especially resistant to alkali and likewise especially sensitive to acid. In view of the curve of hydrogen-ion concentration, it seems that the apparent resistance of the legume bacteria to alkali is due to the slight concentration of hydroxyl-ions in the mannitol solution. From the data of table 1 it will be seen that alfalfa bacteria in mannitol solution



PH
Hydrogen Ion Concentration
of Culture Medium
Inoculated after 2 weeks
Inoculated at beginning
3
4
5
6
7
8
9
10
11
2.77
3.0
3.1
3.6
3.9
6.4
6.8
7.72
8.1
8.4
8.5
8.8
8.9
9.0
9.3
10.1
10.4
11.08
10
7
5
4
3
2
1
0
.5
1
1.5
2
3
C.C. of 0.1 Normal Sodium Hydroxid
C.C. of 0.1 Normal Sulphuric acid

CHART 1

gave the highest count in a neutral solution, although small amounts of alkali had very little effect on the number of bacteria. On the other hand, acid in gram equivalent amounts seriously retarded or prohibited growth. The relation of growth to hydrogen-ion concentration remained almost the same after two and four weeks.

EFFECT OF BACILLUS RADICICOLA ON HYDROGEN-ION CONCENTRATION

Chart 1 gives the curves of the hydrogen-ion concentration of mannitol solution before and after the growth of legume bacteria. The hydrogen-ion concentration is plotted in terms of P_H on the ordinate and the cubic centimeters of acid or alkali on the abscissa. It should be remembered that as P_H increases the concentration of hydrogen-ions decreases and *vice versa*. One of the most striking facts noted by these curves is the difference in the hydrogen-ion concentration of the culture media before and after growth of bacteria. It will be seen that this change is most marked in the case of high P_H of the medium. Apparently the alfalfa bacteria bring about changes in the reaction of the medium which are favorable for their reproduction. The growth of different species of legume bacteria in culture media of varying hydrogen-ion concentration will be discussed in a future paper.

The authors are indebted to Dr. S. F. Acree for many helpful suggestions.

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THE COLON-AEROGENES GROUP FROM SILAGE

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A study of silage fermentation has demonstrated the *colon* organisms to be a predominating type in the earlier stages of ripening. Analyses made from many samples of alfalfa, cane, corn and kafir forage, at time of filling the silo, have given counts ranging from 1000 to 1,000,000 of these organisms per gram of forage. Examinations made from different forage collected in the fields under aseptic conditions showed their presence in numbers ranging from 1000 to 100,000 organisms per gram. This demonstrates that the common forage crops are natural hosts for the colon-aerogenes group, and the large numbers found on the forage as it enters the silo are not due to contamination through handling and cutting, but represent, in a majority of cases, the actual numbers living on the forage.

Numerous investigators have reported the presence of the colon-group on grains, grasses and flowers. They have not agreed, however, as to whether the presence of such organisms signifies fecal contamination or represents a specific type characteristic to grains.

Rogers, Clark and Evans (1915) have examined 166 cultures of coli-like bacteria obtained from dried grains, and demonstrated that none of the types agree with the characteristic flora of the bovine species. Previous to the work of Rogers and his associates, Burri and Andrejew (1910) mentioned that the colon-aerogenes types appearing on grass could be differentiated from the intestinal types. Prescott (1902, 1906) stated that the colon group was able to grow and reproduce on grain, and that there was no distinction between the grain and fecal types.

Preliminary investigations in this laboratory indicate that the colon-aerogenes group commonly present on forage finds there

a favorable environment for growth. Alfalfa was grown in the laboratory where all sources of contamination were prevented except from the soil. The bacteriological examination of the forage gave a count of over 100,000 coli-like organisms per gram. The only possible source of infection from such organisms was through the root system, which does not seem a probable source, or through contamination from the soil as the plants broke through the surface. This latter source is no doubt important.

The purpose of this experiment was to study the physiological activities of the organisms of the colon-aerogenes group present in silage.

METHOD OF PROCEDURE

The number of cultures studied was 110, isolated from the following sources; 95 from various kinds of silage and at different stages of fermentation, of which 53 were from alfalfa silage, 39 kafir silage, and 3 from corn silage. In addition to these 15 strains were obtained from alfalfa and kafir forage which was collected from the fields under conditions as nearly aseptic as possible. Lactose bile in fermentation tubes was used as an enrichment medium. The tubes showing gas were plated on plain and glucose agar, and agar slants were inoculated from representative colonies picked from the plates. All cultures were classified into MacConkey's four groups, according to gas production in glucose, lactose, sucrose, and dulcite broth.

The hydrogen-ion concentration was determined by the method suggested by Clark and Lubs (1915) using methyl red as an indicator. According to their results all grain cultures exhibited a low hydrogen-ion concentration, while that of the fecal strains was high.

The Voges-Proskauer reaction was observed in the medium used for making the hydrogen-ion determination. All cultures were studied as to motility, and action in litmus milk and gelatin.

The temperature of incubation was 37.5°C. for all cultures. The gas production and action in litmus milk were noted after two days, the Voges-Proskauer reaction and hydrogen-ion concentration after four days and the action on gelatin after ten

days' incubation. The gelatin cultures at the end of the incubation period were placed in ice water for several minutes. The failure of the tubes to solidify indicated liquefaction. All cultures showing gelatin liquefaction and absence of gas in lactose were discarded. Motility was observed in twelve hour broth cultures.

EXPERIMENTAL DATA

Table 1 contains a complete summary of the biochemical characteristics of all cultures studied.

TABLE 1

A summary of the physiological activities of the colon aerogenes group from silage

GROUP	TOTAL NUMBER	PER CENT MOTILE	PER CENT ACID TO METHYL RED	PER CENT ALKALINE TO METHYL RED	PER CENT SHOWING VOGES-PROSKAUER REACTION	PER CENT COAGULATING LITMUS MILK
B. coli-communis	12	100.0	100	0	0	100
B. coli-communior	34	55.8	0	100	100	3
B. (lactis) aerogenes	53	64.1	0	100	100	0
B. acidi (lactici)	11	90.9	100	0	0	81.8

The cultures have been classified according to MacConkey's four principal groups, as represented by *B. coli-communis*, *B. coli-communior* *B. (lactis) aerogenes* and *B. acidi (lactici)*.

All cultures of the *B. coli-communis* and *B. acidi (lactici)* groups gave a high hydrogen-ion concentration, which indicates fecal origin, while all cultures of the *B. coli-communior* and *B. (lactis) aerogenes* groups gave a low hydrogen-ion concentration, which signifies grain origin. More recent results obtained, *at this laboratory*, from the study of the colon group from other sources do not, correspond in this respect, however. Neither is there agreement with Greenfield's (1916) work on the colon organisms isolated from water and ice. She found the organisms exhibiting a high and low hydrogen-ion concentration to be distributed in all of MacConkey's four groups.

Browne (1915) states that *B. coli-communis* and *B. acidi (lactici)* are the predominating groups in fresh human feces.

An average of 11 samples gave 31.6 per cent *B. coli-communis*, 57.2 per cent *B. acidi* (*lactici*), 8.3 per cent *B. coli-communior* and 3 per cent *B.* (*lactis*) *aerogenes*. Likewise an average of the 432 cultures isolated by Greenfield from water and ice indicates that 96.3 per cent of the *B. coli-communis* and 91.3 per cent of the *B. acidi* (*lactici*) groups are of fecal origin, while 75.3 per cent of the *B. coli-communior*, and 21 per cent of the *B.* (*lactis*) *aerogenes* groups are of grain origin.

While the figures cited tend to show that the majority of the fecal strains are represented by the *B. coli* (*communis*) and *B. acidi* (*lactici*) groups, not all investigators are in accord.

According to MacConkey's classification the results indicate that 10.9 per cent of the organisms studied were *B. coli-communis* 30.9 per cent *B. coli-communior*, 48.18 per cent *B. lactis* (*aerogenes*) and 10 per cent *B. acidi* (*lactici*). Classified according to origin 79.08 per cent were non-fecal strains, while 20.9 per cent were fecal types.

A direct correlation can be noted between the Voges-Proskauer reaction and hydrogen-ion concentration, which agrees with the results of Levine (1916) and Greenfield. All cultures having a high hydrogen-ion concentration gave a negative Voges-Proskauer reaction, while the converse was true for those cultures having a low hydrogen-ion concentration.

In litmus milk 90.9 per cent of all fecal strains produced acid and coagulation, while 98.5 per cent of the non-fecal types showed only an acid reaction.

While motility of all cultures was determined little importance is attributed to it as a means of classification.

CONCLUSION

1. The study of 95 coli-like cultures isolated from different kinds of silage, and 15 strains obtained from the growing fields of alfalfa and kafir, demonstrates that 48.18 per cent of the organisms are *B.* (*lactis*) *aerogenes*, 30.9 per cent *B. coli-communior*, 10.9 per cent *B. coli-communis* and 10 per cent *B. acidi* (*lactici*).

2. Classified according to origin, as differentiated by methyl red, 79.08 per cent were non-fecal strains, while 20.9 per cent were of fecal origin.

3. All the strains represented by the *B. coli-communis* and *B. acidi* (*lactici*) groups were fecal types, while the organisms included in the groups represented by *B. coli-communior* and *B.* (*lactis*) *aerogenes* were non-fecal strains.

4. A correlation between the Voges-Proskauer reaction and the hydrogen-ion concentration was observed in all cultures.

5. Litmus milk was coagulated by 90.9 per cent of all fecal strains, while 98.5 per cent of the non-fecal types exhibited only an acid reaction.

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